Measurement of the rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate in skeletal muscle by using a single-isotope technique

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The effects of several agents on the rates of the fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle were measured in incubated epitrochlearis muscles of the rat by monitoring the transfer of radiolabel from [6- ^{14}C]glucose to the 1-position of glucose residues in glycogen. The cycling rates observed were almost identical with those previously obtained by using the well-established dual-isotope technique. In particular, it was found that the β -adrenoceptor agonist isoprenaline increased the cycling rate about 12-fold.

The loss of tritium from different positions of the glucose molecule at specific reactions in glycolysis enabled, in principle, rates of substrate ('futile') cycles in glycolysis to be determined. The use of dual-labelled [^3H , ^{14}C]glucose overcame the problem of the need to measure the specific radioactivity of tritium in glycolytic intermediates in small quantities of tissue; thus, by measuring $^3\text{H}/^{14}\text{C}$ ratios in glycolytic intermediates, practical methods for measuring the rates of the glucose/glucose 6-phosphate and fructose 6-phosphate/fructose 1,6-bisphosphate cycles were developed (D. G. Clark *et al.*, 1973; M. G. Clark *et al.*, 1973; for reviews see Katz & Rognstad, 1976; Newsholme & Crabtree, 1976; Hue, 1981). Subsequent studies have found that losses of tritium in the phosphoglucose isomerase reaction and in the triosephosphate isomerase/fructose-bisphosphate aldolase reactions are not complete, so that, to measure precise rates of cycling, it is necessary to separate and measure $^3\text{H}/^{14}\text{C}$ radioactivity ratios in added glucose, glucose 6-phosphate + fructose 6-phosphate and fructose 1,6-bisphosphate (Surholt & Newsholme, 1983; Challiss *et al.*, 1984). This increases markedly the amount of work that has to be carried out to measure the rates of cycling. In addition, the cost of dual-labelled glucose and the requirement for the use of [^3H , ^{14}C]glucose of high specific radioactivity to allow adequate labelling of

the hexose phosphate pools may prevent their use in the study of precise rates of cycling *in vivo*.

A single-isotope method for measuring the rate of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in liver cells was developed by Katz and co-workers (Rognstad *et al.*, 1973; Rognstad & Katz, 1976). This method depends on the fact that some randomization of the upper and lower halves of the glucose molecule occurs in the reaction catalysed by triosephosphate isomerase, and the rate of flux through fructose-1,6-bisphosphatase can be indicated by the randomization of label in hexose monophosphates. Rognstad & Katz (1976) administered [1- ^{14}C]galactose to isolated rat hepatocytes and measured the ratio of ^{14}C specific radioactivities in C-1 and C-6 of the glucose formed. This principle was also used to measure the rate of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in *Escherichia coli* (Chambost & Fraenkel, 1980); however, sufficient randomization of administered [6- ^{14}C]glucose in fructose 1,6-bisphosphate did not occur unless a temperature-sensitive glyceraldehyde-3-phosphate dehydrogenase mutant was used. In the present work, this method was used to measure the rates of this cycle in rat epitrochlearis muscle incubated *in vitro*. In preliminary studies fructose 1,6-bisphosphate was isolated, and and it was shown that randomization of the label between C-6 and C-1

did occur and that it varied according to the incubation conditions, but it was never complete. Nonetheless, sufficient randomization occurred to make this a tenable technique for determination of the rate of this cycle. Since previous studies, with the dual-isotope technique, have obtained precise rates of this cycle in epitrochlearis muscle (Challiss *et al.*, 1984), it is possible to compare directly results from both methods.

Materials and methods

Materials

Animals, chemicals and enzymes were obtained from sources given by Challiss *et al.* (1984); [6- ^{14}C]glucose, [U- ^{14}C]glucose, [U- ^{14}C]fructose 1,6-bisphosphate and $\text{NaH}^{14}\text{CO}_3$ were obtained from Amersham International, Amersham, Bucks., U.K.

Incubation techniques

Animals (male Wistar rats, 150–170 g) were killed by cervical dislocation; epitrochlearis muscles were rapidly removed and muscle pairs pre-incubated for 30 min in 2.5 ml of Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) supplemented with 1.5% (w/v) defatted bovine serum albumin, 4 mM-pyruvate, 5 mM-succinate, 4 mM-glutamate and 5 mM-glucose in an oscillating water bath at 37°C. During this period, flasks were gassed continuously with O_2/CO_2 (19:1). Muscles were then transferred to incubation flasks containing 2.5 ml of Krebs–Ringer bicarbonate buffer supplemented with 1.5% albumin, 5 mM-glucose and 2 μCi of [6- ^{14}C]glucose/ml. Flasks were incubated for 60 min at 37°C with gassing for an initial 15 min period. After 60 min muscle pairs were rapidly blotted and freeze-clamped. Samples of incubation medium were taken for spectrophotometric and radiochemical determination of lactate (Challiss *et al.*, 1983). Glycolytic flux (J) and flux from hexose monophosphate to glycogen (B) were calculated as described by Challiss *et al.* (1984). The measurement of the latter assumes that there is no breakdown of glycogen during the incubation, since some radiolabelled glucose incorporated into glycogen will probably be lost during glycogenolysis. Since some breakdown may occur (see Challiss *et al.*, 1984), the method probably underestimates the rate of cycling.

Isolation and degradation of glycogen

Glycogen was isolated by alkali digestion and ethanol precipitation (Cuendet *et al.*, 1976). About 0.5 mg of carrier glycogen was added, and the precipitation and re-solution in hot water were performed twice. Glycogen was then dissolved in 200 mM-sodium acetate buffer, pH 4.8, containing

8 units of amyloglucosidase and incubated at 40°C for 2 h.

For determination of radioactivity in position 1 of the glucosyl moiety, the method of Chambost & Fraenkel (1980) was used. The pH of the amyloglucosidase digest was adjusted to 7.5 and stabilized by addition of 10 mM- MgCl_2 /100 mM-Tris/HCl buffer, pH 8.2. This was then added to 25 ml Erlenmeyer flasks possessing centre wells containing 2 M-NaOH to collect $^{14}\text{CO}_2$ formed by the 6-phosphogluconate dehydrogenase reaction. To the mixture was added 8 μmol of NADP^+ , 30 μmol of ATP, 3 μmol of oxidized glutathione, 20 μg of glucose-6-phosphate dehydrogenase, 30 μg of 6-phosphogluconate dehydrogenase and 5 μg of glutathione reductase. Incubation vessels were sealed and the reaction was started by the addition of 20 μg of hexokinase. After incubation for 2 h at 30°C, the reaction was terminated by injection of 2.5 M- H_2SO_4 , and samples were incubated for a further 2 h. The contents of the centre wells were spotted on to barium acetate-impregnated filter papers and dried (Dawson, 1977). Discs were counted for radioactivity after addition of scintillation cocktail [0.2% 2,5-diphenyloxazole and 0.005% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] in a Beckman LS7500 scintillation counter, previously programmed with the appropriate quench-correction parameters by using [^{14}C]hexadecane. Because of possible problems associated with counting ^{14}C radioactivity as barium carbonate on filter papers, $\text{NaH}^{14}\text{CO}_3$ was used as a second standardization method. Both standardization procedures yielded similar counting efficiencies (91–95%).

Isolation and degradation of fructose 1,6-bisphosphate

For the measurement of the ratio of ^{14}C in C-1 and C-6 in fructose 1,6-bisphosphate, two to four muscle pairs were pooled, after incubation with [6- ^{14}C]glucose (see above). Fructose 1,6-bisphosphate was isolated from a neutralized HClO_4 extract by the method of Hammerstedt (1980) on Dowex-2 (formate form) ion-exchange columns after addition of 2.5 μmol of carrier fructose 1,6-bisphosphate. After freeze-drying, fructose 1,6-bisphosphate was converted into fructose 6-phosphate by using the enzyme fructose-1,6-bisphosphatase as described by Hammerstedt (1980). After re-chromatography on Dowex-2 (formate form), fructose 6-phosphate was freeze-dried and reconstituted in 10 mM- MgCl_2 /100 mM-Tris/HCl buffer, pH 8.0, and decarboxylation of C-1 was carried out as described above for glycogen glucose residues, except that hexokinase was omitted and the reaction was started by adding 5 μg of phosphoglucose isomerase. The efficiency

of the isolation of fructose 1,6-bisphosphate and its conversion into fructose 6-phosphate was 48–59%. After enzymic removal of ^{14}C at position 1, residual ^{14}C radioactivity was counted and assumed to be entirely in position 6.

Measurement of the rate of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle assuming incomplete randomization of label in fructose 1,6-bisphosphate

The principle of the method used in the present study is that randomization of label from the 6-position in hexose monophosphates is dependent on the activity of fructose-1,6-bisphosphatase. After the administration of $[6\text{-}^{14}\text{C}]\text{glucose}$ to muscle some randomization of ^{14}C between C-1 and C-6 will occur in fructose 1,6-bisphosphate via the near-equilibrium reactions catalysed by triosephosphate isomerase and aldolase. However, it is unlikely that both reactions are sufficiently close to equilibrium for the randomization in fructose 1,6-bisphosphate to be complete. Nevertheless, this lack of randomization can be taken into account provided that the extent of the randomization in fructose 1,6-bisphosphate is known. The formula for calculating the cycling rate under these conditions (see Scheme 1) is derived as described below.

Let the specific radioactivities of ^{14}C in hexose monophosphate (HexP) and fructose 1,6-bisphosphate $[\text{Fru}(1,6)\text{P}_2]$ be S_h and S_d respectively, and the superscripts (1) and (6) refer to ^{14}C in positions C-1 and C-6 of each compound. B , C , F and J are fluxes as defined in Scheme 1. Under steady-state conditions, considering the entry of label into C-1 of hexose monophosphate:

$$C \cdot S_d^{(1)} = F \cdot S_h^{(1)} + B \cdot S_h^{(1)} \quad (1)$$

Considering the entry of total (C-1 + C-6) into fructose bisphosphate:

$$F(S_h^{(1)} + S_h^{(6)}) = (J + C)(S_d^{(1)} + S_d^{(6)}) \quad (2)$$

Scheme 1. Model for substrate cycling at the level of fructose 6-phosphate taking account of an active glycogen/glucose 1-phosphate cycle

HexP represents hexose monophosphate, $\text{Fru}(1,6)\text{P}_2$ represents fructose 1,6-bisphosphate, G , F , C and J represent fluxes through glucose phosphorylation, 6-phosphofructokinase, fructose-1,6-bisphosphatase and glycolysis respectively, and A and B represent the reciprocal fluxes between hexose monophosphate and glycogen.

Let:

$$f = \frac{S_d^{(6)}}{S_d^{(1)}} \quad (3)$$

(If aldolase/triosephosphate isomerase catalyse equilibrium reactions, $f = 1$.)

Substituting eqn. (3) into eqn. (2) and re-arranging eqn. (1) for $S_d^{(1)}$:

$$F(S_h^{(1)} + S_h^{(6)}) = (J + C)(1 + f) \cdot \frac{F + B}{C} \cdot S_h^{(1)} \quad (4)$$

Let:

$$p = \frac{S_h^{(1)}}{S_h^{(6)}} \quad (5)$$

Dividing eqn. (4) through by $S_h^{(6)}$:

$$F(1 + p) = \frac{(J + C)(F + B)(1 + f)p}{C} \quad (6)$$

Substitution of $(J + C)$ for F and re-arrangement:

$$C = \frac{(J + B)(1 + f)p}{(1 - pf)} \quad (7)$$

Results and discussion

The method

The method developed in the present work allows the measurement of flux through fructose-1,6-bisphosphatase with the use of the single radiolabelled substrate $[6\text{-}^{14}\text{C}]\text{glucose}$. The information required is the extent of C-1/C-6 randomization in both fructose 1,6-bisphosphate (f) and hexose monophosphate (p). In the present work, the value of p is obtained from the ^{14}C distribution in the glucose residues of glycogen rather than hexose monophosphate. It was considered that it would be more accurate to obtain the distribution of label in glycogen rather than hexose monophosphates, since a greater amount of radioactivity is present in glycogen and the latter is easier to isolate from extracts of muscle (see Challiss *et al.*, 1984). The technique is dependent on glycogen synthase catalysing the synthesis of glycogen from hexose monophosphates throughout the incubation period, so that there is a continual 'sampling' of the hexose monophosphate pool by glycogen synthase. To ensure sufficient activity of glycogen synthase, insulin was included in all incubations, except for those used to assess basal cycling rates. The results presented in Table 1 indicate that glycogen synthesis occurred in all incubations.

Randomization of label in hexose monophosphates could be achieved by the actions of trans-aldolase and triosephosphate isomerase rather than via fructose-1,6-bisphosphatase activity. However, use of the dual-labelling technique of D.G. Clark *et al.* (1973) involving studies either with

Table 1. Effects of insulin, isoprenaline and propranolol on the rates of cycling between fructose 6-phosphate and fructose 1,6-bisphosphate

Incubations, separations and measurement of radioactivities and calculation of cycling rates were carried out as described in the Materials and methods section. Results are presented as means $\pm$ S.E.M. with numbers of observations in parentheses. J is the glycolytic flux, B is the flux from hexose monophosphate to glycogen and p and f are the C-1/C-6 and C-6/C-1 radioactivity ratios in glycogen and fructose 1,6-bisphosphate respectively (four or five separately incubated muscles were combined for each analysis.) The cycling rate is calculated from the formula:

$$\text{Cycling rate} = (J + B)(1 + f)p/(1 - pf)$$

Conditions	Rates (μmol of glucose residues/h per g)		C-1/C-6 distribution ratio of ^{14}C in glycogen (p)	C-6/C-1 distribution ratio of ^{14}C in fructose 1,6-bisphosphate (f)	Cycling rate ($\mu\text{mol/h per g}$)	
	Glycolysis (J)	Glycogen synthesis (B)			Single-label randomization method	Dual-labelling method*
Basal	3.25 ± 0.20 (12)	0.55 ± 0.04 (12)	0.015 ± 0.003 (10)	2.60 ± 0.25 (3)	0.21	0.22
Insulin	4.55 ± 0.24 (12)	1.48 ± 0.09 (12)	0.026 ± 0.004 (10)	2.38 ± 0.37 (3)	0.56	0.42
Insulin (1 munit/ml) + 0.1 μM -isoprenaline	5.14 ± 0.30 (8)	0.89 ± 0.11 (8)	0.051 ± 0.014 (6)	3.69 ± 0.54 (3)	1.78	1.69†
Insulin (1 munit/ml) + 10 μM -isoprenaline	6.77 ± 0.44 (8)	0.47 ± 0.12 (8)	0.063 ± 0.013 (5)	2.59 (2)	2.79	1.98
Insulin (1 munit/ml) + 10 μM -isoprenaline + 10 μM -propranolol	4.31 ± 0.32 (8)	1.60 ± 0.24 (8)	0.024 ± 0.005 (7)		0.54	0.78

* Data taken from Challiss *et al.* (1984).

† Dual-labelling method result for 0.1 μM -adrenaline.

glucose tritiated in the 3- or 5-position or comparison of cycling rates observed between soleus (a muscle that contains no detectable fructose-1,6-bisphosphatase activity) and epitrochlearis muscles indicates that randomization of label is due only to fructose-1,6-bisphosphatase activity (Challiss *et al.*, 1984).

Two specific points should be noted. First, owing to the low concentration of fructose 1,6-bisphosphate in incubated muscle tissue, it was necessary to combine muscles from a number of separate incubations for analysis of the C-6/C-1 distribution of ^{14}C in fructose 1,6-bisphosphate. Therefore data given in Table 1 are means obtained from combined analyses of 250 mg of epitrochlearis muscle (four or five separate incubations). Secondly, there is a major difference between the determinations of p and f . The analysis of the ^{14}C distribution in C-1/C-6 in glycogen (p) provides a record of the C-1/C-6 randomization throughout the time course of the experiment, whereas that in fructose 1,6-bisphosphate (f) is for that instant in time when the muscle is freeze-clamped. Thus glycogen will contain a proportion of glucose residues incorporated before the establishment of an isotopic steady state.

Cycling rates

The dual-labelling technique for measuring the rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate estab-

lished for the first time precise rates of cycling in muscle. In addition, this study demonstrated that the rate of cycling was increased in incubated epitrochlearis muscle specifically by β -adrenoceptor agonists (Challiss *et al.*, 1984). The results obtained in the present study are presented in Table 1, together with those obtained previously by using the dual-label technique. It is shown that the cycling rate is low under basal conditions ($0.21 \mu\text{mol/h per g}$ of muscle). Although this rate is low in comparison with the maximum glycolytic flux in this muscle, it is consistent with the hypothesis of Newsholme & Crabtree (1976) (see also Newsholme *et al.*, 1984) that the cycling rate will be very low in the resting state, but will be dramatically increased by 'stress' or 'anticipatory' hormones. It is therefore important to note that insulin increased the cycling rate 3-fold, and that the β -adrenoceptor agonist isoprenaline increased the rate of cycling 13-fold (Table 1). The action of isoprenaline is completely abolished by addition of the β -adrenoceptor antagonist propranolol. The cycling rates measured by this method are very similar both qualitatively and quantitatively to those obtained with the dual-isotope technique (Table 1). Hence it is considered that this single-radiolabel technique is satisfactory for measuring precise rates of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle in mammalian muscle. It should therefore provide an alternative method to the dual-labelling technique for determining rates of this cycle, and, coupled with the use of a specific

inhibitor of gluconeogenesis such as 3-mercaptopicolinic acid to inhibit Cori-cycle activity (see Newgard *et al.*, 1984), it might be used to measure the rate of this cycle in the intact animal.

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