Biochem. J. (1982) **206**, 221–229
Printed in Great Britain

Occupancy of phosphorylation sites in pyruvate dehydrogenase phosphate complex in rat heart *in vivo*

Relation to proportion of inactive complex and rate of re-activation by phosphatase

Graham J. SALE and Philip J. RANDLE
Nuffield Department of Clinical Biochemistry, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, U.K.

(*Received 26 January 1982/Accepted 20 April 1982*)

The [γ-^{32}P]ATP-back-titration method of estimating occupancy *in vivo* of the three phosphorylation sites in the pyruvate dehydrogenase complex was improved in precision by specific analysis with trypsin/formic acid, by more effective prevention of site-2 dephosphorylation during purification with NaF, and by other refinements. Disproportionation of phosphorylated complexes during purification was excluded. With this improved method it was shown that the relationship between occupancy of sites and the proportion of complex in the inactive form in rat heart *in vivo* is closely similar to that measured directly in heart mitochondria by incorporation of [^{32}P]P_i. In the heart *in vivo* (as in mitochondria), occupancy of site 1 correlated linearly with the proportion of inactive complex. Occupancy of sites 2 and 3 only approached equivalence to that of site 1 when 99% of the complex was inactive (starved or diabetic rats). When 70% or less of the complex was inactive (resting or exercising fed normal rats), occupancy of sites 2 and 3 was minimal ($3 < 2$) relative to site 1. The initial rate of re-activation by phosphatase of phosphorylated complex from hearts of resting or exercising fed normal rats was approximately three times that of complex from 48 h-starved rats.

Mammalian pyruvate dehydrogenase complexes (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3) are inactivated by phosphorylation and re-activated by dephosphorylation (Linn *et al.*, 1969*a*,*b*). Three sites (serine) are phosphorylated *in vitro* and *in vivo*; the major site of inactivation is site 1, site 2 has no more than a minor role, and phosphorylation of site 3 is non-inactivating (Yeaman *et al.*, 1978; Sugden *et al.*, 1979; Hughes *et al.*, 1980; Sale & Randle, 1981*a*,*b*, 1982). Phosphorylation of sites 2 and 3 can inhibit re-activation by phosphatase (Sugden *et al.*, 1978; Kerbey & Randle, 1979). In rat heart mitochondria, occupancy of phosphorylation site 1 is linearly correlated with the proportion of inactive complex. In the steady state in mitochondria, occupancy of sites 2 and 3 is minimal relative to site 1 ($3 < 2$) when the proportion of inactive complex is 70% or less; occupancy of the three sites approximates to equivalence when complex is completely inactivated (Sale & Randle, 1980, 1982). In rat heart *in vivo* 70% of the complex is inactive in fed normal rats and >99% of the complex is inactive in 48 h-starved or diabetic rats (Kerbey *et al.*, 1976). Occupancy of sites *in vivo* has been estimated by measuring the difference between unoccupied sites and total sites (after dephosphorylation) with [γ-^{32}P]ATP (Sale & Randle, 1981*a*). The results were in broad agreement with those obtained in mitochondria, except that occupancies of site 2 were lower in the heart *in vivo*. Possible reasons for this discrepancy were selective dephosphorylation of site 2 during extraction and purification of complex, and inaccuracies in estimation of site occupancy by tryptic cleavage.

In the present study estimation of site occupancy *in vivo* has been much improved by use of NaF as an additional inhibitor of dephosphorylation and by elimination of the assumptions regarding the relative occupancies of sites 1 and 2 by assay with trypsin/formic acid cleavage (Sale & Randle, 1981*b*, 1982) and by further refinements. The relationship between site occupancy and the proportion of active complex found by this method shows close correspondence to that of mitochondria. It is shown that increased occupancy of sites 2 and 3 in the complex in diabetes and starvation inhibits re-activation by phosphatase.

In this paper phosphopeptides are abbreviated according to the scheme of Sale & Randle (1981*b*, 1982). Trypsin yields TA12 (peptide TA phosphorylated in sites 1 and 2), TA1(A2) (a mixture, of

0306-3283/82/080221-09$01.50/1

TA peptides phosphorylated in either site 1 or site 2) and TB3 (peptide TB phosphorylated in site 3). Formic acid cleaves the TA phospho-tetradecapeptides TA1, TA2 and TA12 into TF1 (residues 1–8, containing site 1) and TF2 (residues 9–14, containing site 2).

Experimental

Materials, rats, assays

Sources of materials, details of assays of pyruvate dehydrogenase complexes and of ^{32}P, and details of rats, were as given in Sale & Randle (1981*a*,*b*, 1982). Additionally, effects of exercise in rats were investigated by 2–5 min of swimming, and those of sodium dichloroacetate (0.15 M; pH 7.0) by intraperitoneal injection 30 min before removing the heart.

Extraction and purification of rat heart pyruvate dehydrogenase phosphate complexes

This was performed by method A of Sale & Randle (1981*a*), modified as follows. Preparations were from ten hearts, and exercised rats (only) were killed by stunning and cervical dislocation before removing the heart. After extraction of complex from frozen powdered hearts (40 ml of extraction buffer), pellets were re-extracted with 2 × 20 ml of buffer. The supernatants were combined and processed and the pellets discarded. Details of buffers were as in Sale & Randle (1981*a*), apart from the inclusion of 20 mM-NaF in all buffers except 2 mM-sucrose buffer, which contained 2 mM-NaF. The final pellet of pyruvate dehydrogenase phosphate was dissolved in the absence of NaF as in Sale & Randle (1981*a*).

Dephosphorylation of rat heart pyruvate dehydrogenase phosphate complexes

Incubations at 30°C were in 25 mM-Tris/HCl/10 mM-potassium phosphate/18 mM-$MgCl_2$/60 μM-$CaCl_2$/2 mM-dithiothreitol (pH 7.0; 10 mM-Mg^{2+}, 43 μM-Ca^{2+}), containing 1–2 units of pyruvate dehydrogenase phosphate complexes/ml. Reaction was initiated with phosphatase (EC 3.1.3.43) after 5 min preincubation at 30°C. For assay of pyruvate dehydrogenase phosphate concentration (100 μl incubation mixture) and for bulk preparation of pyruvate dehydrogenase complex (2–3 ml incubation mixture), sufficient phosphatase was added to effect complete conversion into active complex within 10 min. Incubation was for 30 min and formation of active complex was monitored over this period to ensure that complete conversion had occurred. Bulk preparations of pyruvate dehydrogenase complex were freed of phosphatase by ultracentrifugation as described by Sale & Randle (1981*a*). The same incubation medium was used to measure rates of re-activation of pyruvate dehydrogenase phosphate complexes from hearts of fed and starved rats. Details of the concentrations of phosphatase and times of incubation are given in the Results and discussion section.

Phosphorylation of pyruvate dehydrogenase and pyruvate dehydrogenase phosphate complexes with [γ-^{32}P]ATP

Complexes (0.5 unit/ml) were incubated for 30 min at 30°C in 20 mM-potassium phosphate/10 mM-EGTA / 100 mM-NaF / 2 mM-dithiothreitol / 1 mM-$MgCl_2$/0.5 mM-[γ-^{32}P]ATP (200 c.p.m./pmol). Incorporation of ^{32}P was complete in 10–20 min (assayed as in Sale & Randle, 1981*a*). Incorporation of ^{32}P into individual sites was determined by a combination of tryptic digestion and formic acid cleavage of phosphopeptide TA12 after electrophoretic separation as described by Sale & Randle (1981*b*, 1982). Tryptic phosphopeptide TA1(A2) was essentially absent (2 ± 0.6% in pyruvate dehydrogenase complex preparations; 0% in pyruvate dehydrogenase phosphate complex preparations), confirming that phosphorylation was complete.

Calculations

One unit of pyruvate dehydrogenase complex forms 1 μmol of NADH/min at 30°C. Site occupancies in inactive pyruvate dehydrogenase phosphate complexes were calculated as described by Sale & Randle (1981*a*), modified to include analysis of phosphopeptide TA12 by formic acid cleavage (Sale & Randle, 1981*b*, 1982). In calculating overall site occupancies (i.e. in both active and inactive forms), zero occupancy of sites in active complex was used.

The concentrations of Mg^{2+} and Ca^{2+} were calculated as described by Severson *et al.* (1974), making no allowance for traces of Ca or Mg in the phosphatase preparation or traces of EDTA in the pyruvate dehydrogenase phosphate complex preparations.

Results and discussion

Improvements in extraction and purification of rat heart pyruvate dehydrogenase phosphate complex

The [γ-^{32}P]ATP-back-titration method for measurement of site occupancies *in vivo* necessarily involves purification of phosphorylated complex to remove ATPases and other interconvertible enzymes and their kinases and phosphatases. For the method to be valid, purified phosphorylated complexes must be representative of the total pool in the heart, and interconversion must be prevented during extraction and purification. Evidence on these points is as follows.

Optimum conditions for extraction of phosphorylated and active complex involves the use of 50 ml of extraction buffer/g of heart powder. For

purification of complex, the volume of extraction buffer was necessarily less (8ml/g). The improved procedure used in the present study extracted all phosphorylated complexes, but only 10% of active complex. This loss of active complex was 27% or 59% of total complex in resting or exercising fed normal rats respectively, and <1% in starved or diabetic rats. Representative data are in Table 1. Recovery of active complex was not improved by proteinase inhibitors [ox serum, leupeptin, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK')]. Triton X-100 extracted approx. 80% of active complex, but activity was lost during poly(ethylene glycol) fractionation. Loss of active complex may only lead to errors in measurement of site occupancy if it becomes inactivated by proteolysis or denaturation but is phosphorylated by [γ-^{32}P]ATP. This is unlikely, because (*a*) there was negligible incorporation of ^{32}P into site 1 of inactive complexes (<2% of all site 1), (*b*) lysosomal proteinase(s) release phosphorylation sites in pyruvate dehydrogenase (EC 1.2.4.1) which is separated from holocomplexes by centrifugation at 150000*g* for 1.5h (Kresze & Steber, 1979), and (*c*) after dephosphorylation, incorporations of ^{32}P (nmol of P/unit of complex inactivated) were closely similar, being 2.00 (fed normal), 1.95 (exercised fed normal), 1.89 (starved) and 2.02 (diabetic). The presence of 27% or 59% of inactive dephosphorylated complex (resting or exercising fed normal) would increase ^{32}P incorporation by 37% or 144% respectively if fully phosphorylated. We conclude that loss of active complex is unlikely to be a significant source of error.

The recovery of phosphorylated complexes after purification was approx. 40% (Table 1). Because phosphorylated complexes in the heart may be heterogeneous with respect to site occupancy, it was important to ascertain whether disproportionation of species of phosphorylated complexes occurred during purification. This was assessed by co-purification of [^{32}P]phosphorylated complexes. Powdered frozen rat heart was mixed before extraction with powdered frozen solutions of [^{32}P]phosphorylated pig heart complexes or with powdered frozen rat heart mitochondria containing [^{32}P]phosphorylated complexes. Two pig heart [^{32}P]phosphorylated complexes were used with relative occupancies (site 1:site 2:site 3) of 1:0.22:0.05 and 1:0.84:0.33. After co-purification relative occupancies were 1:0.24:0.05 and 1:0.84:0.32. Rat heart mitochondria were incubated with [^{32}P]P_i/2-oxoglutarate/L-malate for 10min; sodium dithionite was then added to 10mM and incubation continued for a further 10min as described by Sale & Randle (1982). Relative occupancies in the complex in mitochondria (site 1:site 2:site 3) were 1:0.42:1.37 (this procedure was chosen because it gives a relative excess of site-3 occupancy; Sale & Randle, 1982). After co-purification relative occupancies were 1:0.43:1.24. If disproportionation occurred during co-purification, the relative occupancy of phosphorylation sites would be altered; relative occupancies of initial and co-purified [^{32}P]phosphorylated complexes were closely similar. In tryptic/formic acid analysis the only loss noted was of TF2 from TA1(A2), i.e. of peptide chains phosphorylated in site 2 but not site 1. Under steady-state conditions this type of complex only accounts for 0.7% of inactive complexes in heart mitochondria (Sale & Randle, 1982). Its loss is thus unlikely to affect significantly estimation of site occupancy *in vivo*. It was further shown that co-purified ^{32}P-labelled partially phosphorylated complex could be phosphorylated to completion on incubation with non-radioactive ATP [assayed by disappearance of TA1(A2)], i.e. the kinase reaction is fully preserved. The results of experiments described in this paragraph show also that phosphorylation or dephosphorylation of complexes does not occur during extraction or purification.

The control experiments described above are

Table 1. *Yields of active pyruvate dehydrogenase complex and inactive pyruvate dehydrogenase phosphate complex during extraction and purification*

The values in parentheses at the head of each column are those obtained under conditions optimal for extraction and assay of pyruvate dehydrogenase complex (active + inactive forms) in rat heart (see Sale & Randle, 1981*a*). Recoveries elsewhere in the Table are based on these values. The extraction and purification procedures were as described in the Experimental section.

		Activity of complex (units/g wet wt.)			Recovery (%)	
Fraction	Step	Phosphorylated	Active	Active complex (% of total complex)	Phosphorylated	Active
		(2.95)	(1.15)	(28.0)	(100)	(100)
1	Bulk extraction	3.20	0.10	3.0	108	9
2	Solubilized poly(ethylene glycol) precipitate	2.65	0.08	2.9	90	7
3	Final preparation	1.18	0.03	2.2	40	3

more stringent than those used previously (Sale & Randle, 1981*a*). In the present study [^{32}P]phosphorylated complexes were added before extraction; in the earlier study they were added to the extract. In the present study experiments with [^{32}P]phosphorylated complexes in mitochondria were additionally included. This is important, because extraction of complex from rat heart is extraction of complex from rat heart mitochondria. As described in more detail in the next section, the procedure in the present study was much more effective in preventing dephosphorylation of site 2.

Occupancies of phosphorylation sites in vivo

Total incorporations of ^{32}P from [γ-^{32}P]ATP. In the present study [^{32}P]phosphorylation of available sites in phosphorylated complexes was complete by criteria given in Sale & Randle (1981*a*). Incorporation into dephosphorylated complexes was 1.94 ± 0.02 nmol of P/unit of complex inactivated (mean ± S.E.M. for all hearts). The ratios of ^{32}P in TF1/TF2 from TA12 and in TA12/TB3 were 0.95 ± 0.01 and 2.07 ± 0.02 (mean ± S.E.M. for 11 observations), showing that dephosphorylation was complete. Incorporations into purified pyruvate dehydrogenase phosphate complexes were lower, being 0.69 ± 0.02 (fed normal; 25 observations on four preparations), 0.65 ± 0.03 (exercised fed normal; five observations on one preparation), 0.13 ± 0.02 (48h starved; 19 observations on three preparations) and 0.13 ± 0.01 (alloxan-diabetic; five observations on one preparation) (mean ± S.E.M., in nmol of P/unit of complex). The percentages of available sites in inactive complexes phosphorylated *in vivo* were thus 65 ± 0.9 (fed normal), 66 ± 0.8 (exercised fed normal) and 94 ± 1.0 (starved or diabetic). Starvation and alloxan-diabetes thus increased ($P < 0.001$) total occupancy of phosphorylation sites in inactive complex *in vivo* compared with fed normal controls, in addition to increasing the proportion of inactive complex (from 72% to 99%). Exercise in fed normal rats (2min of swimming) decreased the proportion of inactive complex from 72% to 37.5%, but did not change total site occupancy in the inactive complex. The new proportion of inactive complex was steady state; the effect of 5min of exercise was comparable. Similar results were obtained when the steady-state proportion of inactive complex was decreased by intraperitoneal injection of sodium dichloroacetate (kinase inhibitor) (results not shown).

Occupancy of site 1. ^{32}P incorporations into individual phosphorylation sites have been determined by using a combination of tryptic digestion (TB3; ^{32}P in site 3) and formic acid cleavage of tryptic phosphopeptide TA12 into TF1 (^{32}P in site 1) and TF2 (^{32}P in site 2). Control cleavages of peptide TA12 from dephosphorylated complexes phosphorylated with [γ-^{32}P]ATPMg gave a ^{32}P ratio TF1/TF2 of 0.95 ± 0.01 (9). Cleavages of TA12 from pyruvate dehydrogenase phosphate complexes phosphorylated with [γ-^{32}P]ATP gave ^{32}P ratios TF1/TF2 of 0.0007 ± 0.0007 (3) (exercised or sodium dichloroacetate-treated), 0.0196 ± 0.007 (3) (fed normal), or 0.42 ± 0.12 (3) (alloxan-diabetic or starved). In absolute terms these values correspond to incorporations into TF1 of (nmol of P/unit) 0.0002 ± 0.0001, 0.0055 ± 0.0009 and 0.025 ± 0.005, i.e. the occupancies of site 1 in the inactive pyruvate dehydrogenase phosphate complexes are 99.9 ± 0.9% (exercised or dichloroacetic acid-treated), 99.2 ± 0.9% (fed normal) and 96.3 ± 0.9% (starved or alloxan-diabetic). These results show that the inactive complexes as prepared contain site 1 essentially fully occupied with non-radioactive phosphate and validate the assumption of Sale & Randle (1981*a*). The small incorporations into TF1, especially in starved or alloxan-diabetic animals, appear to represent turnover of unlabelled phosphate for ^{32}P (results not shown).

Occupancies of sites 2 and 3. In all preparations of pyruvate dehydrogenase phosphate complex from the hearts of starved or alloxan-diabetic rats, the occupancies of sites 2 and 3 were always significantly higher than in complex from the hearts of normally fed rats (Table 2) ($P < 0.001$, all differences); occupancies of site 1 in all cases approached 100% and were not significantly different (see above). Combined values for percentage site occupancies in inactive complex *in vivo* for these different types of rat heart were (site 1, 2, 3): 99.3 ± 1.1, 56.6 ± 1.5, 37.1 ± 1.3 (fed normal, 25 observations on four preparations); 96.0 ± 3.0, 95.0 ± 1.2, 90 ± 1.0 (alloxan-diabetic, five observations on one preparation); 96.5 ± 1.5, 94.5 ± 1.7, 86.5 ± 2.0 (48h-starved, 19 observations on three preparations). The occupancies of site 3 are similar to those determined previously (Sale & Randle, 1981*a*). Occupancies of site 2 are higher; this may be attributed to more efficient inhibition of dephosphorylation during preparation of inactive complex by inclusion of 20mM-NaF (in addition to 50–10mM-EDTA) in the present work. Dephosphorylation may lead to an underestimation of site-2 occupancy relative to sites 1 or 3. This is because site 2 is dephosphorylated 2–3 times faster than sites 1 or 3 by pyruvate dehydrogenase phosphate phosphatase (Sale & Randle, 1982). Co-purification experiments (see the preceding section) confirmed the absence of dephosphorylation in the present work. Co-purification experiments by Sale & Randle (1981*a*) indicating lack of dephosphorylation in the absence of NaF may have been less stringent (because ^{32}P-labelled complex was not added before extraction, but after centrifugation of the initial extract). A further factor may be turnover of unlabelled phos-

Table 2. *Occupancy in vivo of sites of phosphorylation in inactive pyruvate dehydrogenase phosphate complexes*
Preparations (1, 3, 6), (2, 5, 10), (7, 9, 11) and (4, 8) were processed and analysed simultaneously. The percentage of complex in the inactive form *in vivo* was determined as in Table 1. Site occupancies were estimated by back titration of unoccupied sites in inactive complex with [γ-^{32}P]ATP, total sites being estimated with [γ-^{32}P]ATP after complete dephosphorylation with phosphatase. Separate incorporation of ^{32}P into sites 1, 2 and 3 was determined by trypsin/formic acid cleavage. For further details see the Experimental section. Occupancies (%) are given as means ± S.E.M. for not less than six observations on each preparation. $^*P < 0.001$ for differences from normal fed, or exercised normal fed, or dichloroacetate-treated normal fed.

Preparation no.	Rats	Percentage of complex in inactive form *in vivo*	Occupancy of sites (%) in phosphorylated inactive complexes		
			Site 1	Site 2	Site 3
1	Diabetic, fed	99.0	96.0 ± 3.0	95.0 ± 1.2*	90.0 ± 1.0*
2	Normal, starved	99.5	95.2 ± 2.8	96.7 ± 3.8*	88.6 ± 2.8*
3	Normal, starved	99.0	98.0 ± 3.0	92.0 ± 3.0*	78.0 ± 3.1*
4	Normal, starved	99.6	96.3 ± 2.5	94.8 ± 2.6*	91.2 ± 2.7*
5	Normal, fed	70.8	98.8 ± 1.6	56.9 ± 1.3	36.7 ± 1.3
6	Normal, fed	72.0	99.5 ± 3.1	48.0 ± 3.5	40.0 ± 3.6
7	Normal, fed	71.5	99.2 ± 3.4	62.9 ± 3.1	37.1 ± 3.3
8	Normal, fed	67.5	99.7 ± 1.1	57.1 ± 1.9	35.6 ± 2.3
9	Normal, fed, dichloroacetate (0.32 μmol/g)	50.0	100.5 ± 2.0	62.6 ± 2.2	47.1 ± 2.3
10	Normal, fed, exercised	37.5	100.6 ± 2.1	53.7 ± 2.2	41.4 ± 2.6
11	Normal, fed, dichloroacetate (0.35 μmol/g)	33.0	100.0 ± 4.1	51.2 ± 3.6	35.1 ± 3.9

phate for ^{32}P at a rate not detectable as statistically significant in plots of total ^{32}P incorporation against time; this may occur when phosphorylations are performed in the absence of NaF, as in the previous work. In the present study phosphorylations were performed in the presence of 100 mM-NaF and this suppressed turnover to very low values, which were (in nmol of P/30 min) for site 1 (assayed by formic acid cleavage of TA12) 0.0002 (exercised or dichloroacetate-treated), 0.006 (fed normal), 0.025 (alloxan-diabetic or starved). Turnover is assumed to be due to residual phosphatase activity, and may be expected to occur at site 2 at a rate 2–3 times more rapidly than at sites 1 or 3. Tryptic digestion alone was used in the earlier study and ^{32}P incorporation into TA12 (turnover at sites 1 and 2 + phosphorylation of unoccupied sites) was assumed to be into site 2. This may also have led to underestimation of the occupancy of site 2.

The effect of decreasing the proportion of inactive complex below 72% was investigated by exercise (which may operate predominantly by Ca^{2+}-mediated increase in phosphatase activity; Hiraoka *et al.*, 1980; McCormack & Denton, 1981) and by injection of dichloroacetate (which inhibits the kinase reaction; Whitehouse *et al.*, 1974). Decreasing the proportion of inactive complex in fed rats from 72% to 55–33% by either method did not appreciably change relative occupancies of phosphorylation sites *in vivo*.

Relationship between overall occupancy of phosphorylation sites and proportion of complex in the inactive form in the rat heart in vivo

With all categories of heart tested, values of this relationship for each site could be fitted to a single line (Fig. 1). Overall occupancy of site 1 was linearly correlated with the proportion of inactive complex; the slope and intercept (mean ± S.E.M.) were 1.04 ± 0.01 and −1.4 ± 0.9 respectively and the correlation coefficient (r) was 0.99. Overall occupancies of sites 2 and 3 were not correlated linearly with the proportion of inactive complex. Between 33 and 72% of inactive complex, occupancies were lower than that of site 1 (site 3 < site 2) and showed little relative change. Between 72% and 99% of inactive complex occupancy of site 2 (×1.7) and site 3 (×2.4) increased relative to site 1 to approach equivalence at >99% of inactive complex. Taken together, the results show that starvation or alloxan-diabetes increases the proportion of inactive complex in rat heart (×1.4) and that this is associated with net increases in overall occupancy for sites 1, 2 and 3 of 1.4-, 2.5- and 3.4-fold respectively.

The profiles in Fig. 1 are closely similar to those obtained in rat heart mitochondria in which the proportion of inactive complex was varied by increasing Ca^{2+} or pyruvate concentration (Sale & Randle, 1980, 1982). At 70% inactive complex, relative occupancies (site 1 : site 2 : site 3) were

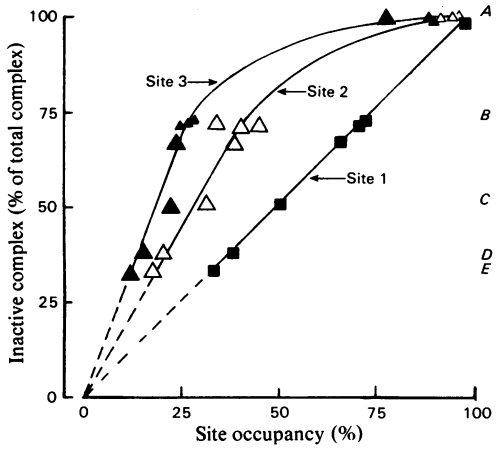

Fig. 1. *Relationship between the proportion of pyruvate dehydrogenase complex in the inactive form and the overall occupancies of phosphorylation sites 1* (■), *2* (△) *and 3* (▲) *in rat heart in vivo*

The percentages of complex in the inactive form were determined as in Table 1. Site occupancies were determined as in Table 2 and were corrected for active complex present in the heart *in vivo* (zero occupancy) to give overall occupancy. Hearts from: *A*, 48h-starved or alloxan-diabetic rats; *B*, normal fed rats; *C*, normal fed dichloroacetate-treated (0.32 μmol/g) rats; *D*, normal fed exercised rat; *E*, normal fed dichloroacetate-treated (0.35 μmol/g) rats.

1:0.6:0.4 (mitochondria incubated with Ca^{2+}), 1:0.7:0.3 (mitochondria incubated with pyruvate), and 1:0.6:0.4 (heart *in vivo*, present study). At 99% inactive complex the corresponding ratios were 1:1:1 (mitochondria) and 1:1:0.9 (*in vivo*). These results may show that site 1 is the major inactivating site *in vivo* as in purified complex (Sale & Randle, 1981*b*) and rat heart mitochondria (Sale & Randle, 1982).

Effect of occupancy of phosphorylation sites 2 and 3 in pyruvate dehydrogenase phosphate complex in vivo on the rate of re-activation by phosphatase

The initial rate of re-activation by phosphatase of partially phosphorylated pig heart complex (relative occupancies sites 1:2:3, 1:0.14:0.03) was 5–6 times that of fully phosphorylated complex (relative occupancies 1:0.94:0.78) (Kerbey & Randle, 1979). The objective of the present experiments was to quantify the effect of increased phosphorylation of sites 2 and 3 in rat heart complex (induced *in vivo* by 48h starvation) on the rate of re-activation by phosphatase. Dependency on phosphatase concentration has also been examined. In the experiments of Kerbey & Randle (1979), the ratio of initial rates of re-activation was calculated from the intercept (zero re-activation) of least-squares linear-regression analysis of the re-activation ratio (partially phosphorylated complex/fully phosphorylated complex) against percentage re-activation of the partially phosphorylated complex. This empirical method of analysis is useful for combining data at different phosphatase concentrations. Application of this method of analysis to the results of the present study gave a ratio of initial rates of re-activation (fed/48h-starved) of 2.82 ± 0.21 (mean ± S.E.M. for 42 observations; $P < 0.001$ for difference from unity). In this analysis the ratios for (resting fed/starved) and (exercising fed/starved) did not differ significantly and have been combined.

In the present experiments, phosphorylated complexes were purified from hearts of 48h-starved rats (two preparations), resting fed rats (two preparations) and exercising fed rats (one preparation). The occupancies of sites of phosphorylation are given in the legend to Table 3. Re-activation was followed at three different concentrations of phosphatase (two preparations were used in separate experiments). The composition of the incubation medium is given in the Experimental section; the concentrations of phosphatase, phosphorylated complexes and times of incubation are given in Table 3. The data are summarized in Table 3 as apparent first-order rate constants for the re-activation of individual preparations of phosphorylated complex by each concentration of phosphatase. The apparent first-order rate constants were calculated by least-squares linear-regression analysis of ln[(concentration of inactive complex at zero time)/(concentration of inactive complex at time t)] against time of incubation (t). The validity of this method of analysis is shown by the correlation coefficient (r) (>0.95 in each case), the absence of significant deviations from linearity ($P > 0.1$ in each case), and the close agreement between the intercept ($t = 0$) and the observed zero-time concentration of inactive complex (given in the legend to Table 3).

The data show that the ratio of rates of re-activation of inactive complexes for (fed resting/starved resting) is independent of phosphatase concentration over the range employed. The overall mean for the ratio of rates of re-activation was 2.77 ± 0.15 (mean ± S.E.M. for 84 observations). For (fed exercising/starved resting) the ratio at the lowest phosphatase concentration was possibly lower ($0.05 > P > 0.02$) than at the highest phosphatase concentration. The overall mean for the ratio of rates re-activation was 2.40 ± 0.07 (mean ± S.E.M. for 40 observations). These data are shown in columns 6 and 7 of Table 3.

At the phosphatase concentrations employed (see Table 3), the rate constants for re-activation of fully

Table 3. *Rates of re-activation by phosphatase of phosphorylated pyruvate dehydrogenase complexes purified from hearts of fed or 48 h-starved rats*

Phosphorylated complexes were purified and site occupancy was estimated by methods given in the Experimental section. The site occupancies are given in Table 2 (preparations 2, 5 and 10 in Expt. A; preparations 4 and 8 in Expt. B). Two phosphatase preparations were used, one in Expt. A and one in Expt. B. Incubations were as described in the Experimental section (computed Mg^{2+} 10mM, Ca^{2+} 43μM) and relative phosphatase concentrations in each experiment are shown in this Table. Initial concentrations of phosphorylated complex (units/ml) were 1.12 (Expt. A) and 1.61 (Expt. B). Formation of active complex was monitored at intervals which ranged from 17s to 40min depending on activity of phosphatase. Except for 6% phosphatase in Expt. A, complete re-activation was achieved in all incubations. Apparent first-order rate constants were calculated by least-squares linear-regression analysis of ln[(concentration of inactive complex at time 0)/(concentration of inactive complex at time t)] against t. Deviations from linearity were not significant, and the correlation coefficients (r) ranged from 0.96 to 0.99. The intercept at $t = 0$ (which should be 0) was 0.01 ± 0.03 (mean ± S.E.M. for all values). Values in the Table are means ± S.E.M. for the numbers of time points shown in parentheses.

		Rate of re-activation of inactive complex; apparent first-order rate constant (min^{-1}) for rats:			Ratio of rate constants	
					Fed (resting)	Fed (exercising)
Expt.	Phosphatase concentration (%, v/v)	Fed (resting)	Fed (exercising)	48h-starved (resting)	Starved (resting)	Starved (resting)
A	50	0.51 ± 0.04 (11)	0.56 ± 0.06 (6)	0.20 ± 0.02 (8)	2.53 ± 0.23	2.75 ± 0.31
	29	0.19 ± 0.01 (4)	0.22 ± 0.01 (5)	0.09 ± 0.02 (4)	2.10 ± 0.34	2.39 ± 0.37
	6	0.05 ± 0.01 (8)	0.04 ± 0.01 (5)	0.02 ± 0.001 (7)	2.63 ± 0.16	1.90 ± 0.07
B	29	3.24 ± 0.36 (5)	—	0.90 ± 0.18 (6)	3.60 ± 0.66	—
	13	1.97 ± 0.42 (4)	—	0.69 ± 0.06 (5)	2.86 ± 0.51	—
	2.8	0.31 ± 0.04 (6)	—	0.11 ± 0.01 (6)	2.93 ± 0.33	—

phosphorylated complexes (from starved rats) ranged from 0.02 ± 0.001 to 0.90 ± 0.18 (lines 3 and 4 of column 5 of Table 3). In rat heart mitochondria the rate constants for re-activation of fully phosphorylated complex ranged from 0.07 ± 0.004 (turnover) to 0.43 ± 0.07 (10mM-sodium dithionite/0.5μM-Ca^{2+}) (see column 2 in Table 3 of Sale & Randle, 1982). Thus the range of rates of re-activation in the present study encompasses the range of rates observed in mitochondria.

General discussion and conclusions

There is evidence that phosphorylation of either site 1 or site 2 inactivates purified pyruvate dehydrogenase complexes (Teague *et al.*, 1979; Radcliffe *et al.*, 1980; Tonks *et al.*, 1982). It has been suggested that the function of site 2 is as an inactivating site additional to site 1 (Reed *et al.*, 1980; Reed & Pettit, 1981). However, the contribution of site 2 to inactivation is <2% during phosphorylation (purified complex, or complex in rat heart mitochondria) and <1% in the steady state in mitochondria (Sale & Randle, 1981*b*, 1982). The major function of site 2 may therefore lie elsewhere. The contribution of site 2 to inactivation can increase to 7% during dephosphorylation of fully phosphorylated complex in mitochondria, i.e. it may inhibit re-activation by phosphatase (Sale & Randle, 1982). Site 3 is apparently not inactivating, and its function must also lie elsewhere (Reed *et al.*, 1980; Reed & Pettit, 1981; Sale & Randle, 1980, 1982).

In both rat heart mitochondria (Sale & Randle, 1980, 1982) and in the heart *in vivo* (the present study), the proportion of complex in the inactive form was correlated linearly with occupancy of site 1. Occupancy of sites 2 and 3 relative to site 1 (2 > 3) was minimal when <70% of complex was inactive and approached equivalence to site 1 only when >99% of complex was inactive. *In vivo* the interconversion system in heart mitochondria would appear to operate such that minimal occupancy of sites 2 and 3 relative to site 1 is achieved in resting or exercising fed normal rats and maximal occupancy is achieved in starved or diabetic rats. Qualitatively this behaviour is that expected from consideration of the relative rates of phosphorylation in active complexes (site 1 > 2 > 3) and of the relative rates of dephosphorylation in fully phosphorylated complexes (site 2 > 1 = 3) (Yeaman *et al.*, 1978; Teague *et al.*, 1979; Kerbey *et al.*, 1979, 1981; Sale & Randle, 1981*b*, for purified complexes) (see also Sale & Randle, 1980, 1982, for complex in mitochondria). Equivalent occupancies of the three sites may be expected only at a high activity ratio of kinase/phosphatase, such as is seen in starvation and diabetes (Hutson & Randle, 1978; Randle *et al.*, 1981; Kerbey & Randle, 1981).

Phosphorylation of sites 2 and 3 in purified

complexes was found to inhibit re-activation by phosphatase (by up to 5–6-fold) by Sugden *et al.* (1978), Kerbey & Randle (1979), Sugden & Simister (1980) and Kerbey *et al.* (1981). These findings have been criticized on the grounds that the concentration ratio (phosphatase/phosphorylated complexes) was low in relation to the ratio in mitochondria, and of failure to confirm the findings with phosphatase purified to apparent homogeneity (Reed & Pettit, 1981). This latter criticism perhaps begs the question as to whether some mitochondrial factor is lost, when phosphatase is highly purified, which may be involved in differential rates of re-activation of partially and fully phosphorylated complexes. Criticisms based on concentration ratio (phosphatase/phosphorylated complex) may be unjustified. Evidence has been given that phosphorylation of sites 2 and 3 in the complex in mitochondria is associated with a diminished rate of re-activation by phosphatase (Sale & Randle, 1980). It is moreover difficult to make simple comparisons between experiments with purified complexes (which are solubilized) and complex in mitochondria (which appears to be membrane-bound; Randle & Denton, 1973; Stanley & Perham, 1980). This is one reason why it is important to make comparisons between interconversions in the purified complex, in complex in mitochondria and in complex *in vivo*; it is encouraging to find evidence for comparability in the three systems.

In the present study the ratio of rates of re-activation of partially and fully phosphorylated complexes from hearts of fed and starved rats respectively was 2.8. This ratio was independent of phosphatase concentration over a range which gave rates of re-activation comparable with those seen in mitochondria. Hutson *et al.* (1978), on the basis of experiments with heart mitochondria, suggested that increased phosphorylation of sites additional to the inactivating site might inhibit re-activation by phosphatase in starved or diabetic rats. The results of the present study have provided direct evidence for this suggestion; increased occupancies of phosphorylation sites 2 (×1.8) and 3 (×2.4) relative to site 1 induced *in vivo* by starvation decreased the initial rate of re-activation (×0.37) by partially purified phosphatase.

Note Added in Proof (Received 1 April 1982)

Since submitting this paper, we have had an opportunity to analyse occupancy of phosphorylation sites in complex in hearts perfused with [^{32}P]P_i. This is the result of a generous gift of hearts and immunoprecipitates of pyruvate dehydrogenase complex therefrom by Dr. R. M. Denton, Dr. P. J. England and Dr. T. J. Hopkirk of the Department of Biochemistry, University of Bristol. This technique was not appropriate for our studies *in vivo*, but the collaboration has enabled us to show that occupancies measured by [^{32}P]P_i incorporation in perfused heart are in accord with those obtained by back titration *in vivo*.

Hearts from fed rats (eight in each group) were perfused by Dr. P. J. England (7kPa; 20min; 38°C) with medium containing (initially) 10mM-glucose and 0.23mM-[^{32}P]P_i (319d.p.m./pmol), with or without a further 2min of perfusion with 0.1μM-isoprenaline, and freeze-clamped at liquid-N_2 temperature (England, 1975). The proportion of inactive complex measured by Dr. T. J. Hopkirk was 85 ± 1% (no isoprenaline) or 51.5 ± 2% (with isoprenaline) (mean ± S.E.M. for eight hearts). The [^{32}P]phosphorylated complex was isolated either by immunoprecipitation of samples of individual hearts (Hughes *et al.*, 1980) or by the purification procedure described in the Experimental section of the present paper (by pooling the residual samples). Occupancies (^{32}P) were then determined by tryptic digestion followed by formic acid cleavage of TA1(A2) after electrophoretic separation.

The results are the means either of single analyses of eight samples (immunoprecipitates) or of three analyses of a single sample (purification procedure). The S.E.M. values were 0.01, except for site 3 of isoprenaline-treated purified complex, where it was 0.02. Relative occupancies (site 1:site 2:site 3) were: without isoprenaline 1:0.61:0.24 (immunoprecipitate) and 1:0.63:0.24 (purified); with isoprenaline 1:0.53:0.24 (purified). Immunoprecipitates of hearts exposed to isoprenaline were contaminated with unidentified [^{32}P]phosphorylated proteins (shown by Dr. T. J. Hopkirk by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis). Because of this contamination electrophoretograms of tryptic digests were not interpretable. For hearts without isoprenaline the results show that relative occupancies (^{32}P) in immunoprecipitates or purified complexes were not significantly different.

For all hearts the values for relative occupancies of sites 1 and 2 were on the lines shown in Fig. 1. The values for site 3 were lower; the reason is not known, but it is suggested that equilibration of site-3 phosphate with ^{32}P in the γ-phosphate moiety of ATP in the heart was incomplete. The specific radioactivity of γ-^{32}P in ATP in the heart is not steady state; after 15min of perfusion it is approx. 30% of that of the [^{32}P]P_i in the perfusion medium at that time (P. J. England, personal communication). Cleavage of TA12 phosphopeptides with formic acid afforded an opportunity to examine whether site-1 phosphate and site-2 phosphate were equilibrated to an equivalent extent. The ratio of [^{32}P]TF1/[^{32}P]-TF2 was 0.90 ± 0.04 (mean ± S.E.M.; eight observations), suggesting that the two sites were equili-

brated to equivalence. It is known that turnover of site 3 in rat heart mitochondria can be slower than that of sites 1 and 2 (Sale & Randle, 1982). The alternative explanation, that site 3 in pyruvate dehydrogenase phosphate was incompletely phosphorylated in the back-titration method, seems unlikely for reasons given in the main body of the paper.

Formic acid cleavage of TA1(A2) from immunoprecipitates of hearts without isoprenaline showed that 98.2% of ^{32}P was in site 1 and 1.8% in site 2. On the basis of arguments given in Sale & Randle (1982), 99.2% of species of phosphorylated complexes were inactivated by phosphorylation of site 1 and 0.8% by phosphorylation of site 2 alone. The contribution of site 2 to inactivation *in vivo* was thus small, in agreement with the findings in rat heart mitochondria (Sale & Randle, 1982).

This work was supported by grants from the Medical Research Council and the British Diabetic Association.

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