Role of individual phosphorylation sites in inactivation of pyruvate dehydrogenase complex in rat heart mitochondria

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1. A method is described using trypsin/formic acid cleavage for unambiguously measuring occupancies of phosphorylation sites in rat heart pyruvate dehydrogenase [^{32}P]phosphate complexes. 2. In mitochondria oxidizing 2-oxoglutarate + L-malate relative initial rates of phosphorylation were site 1 > site 2 > site 3. 3. Dephosphorylation and reactivation of fully phosphorylated complex was initiated in mitochondria by inhibiting the kinase reaction. Using dichloroacetate relative rates of dephosphorylation were site 2 > (1 = 3). Using sodium dithionite or sodium pyruvate or uncouplers + sodium arsenite or steady state turnover (^{31}P replacing ^{32}P in inactive complex) relative rates were site 2 > site 1 > site 3. With dithionite reactivation was faster than site 3 dephosphorylation, i.e. site 3 is apparently not inactivating. 4. The steady state proportion of inactive complex was varied (92–48%) in mitochondria oxidizing 2-oxoglutarate/L-malate by increasing extramitochondrial Ca^{2+} (0–2.6 μM). This action of Ca^{2+} induced dephosphorylation (site 3 > site 2 > site 1). These experiments enable prediction of site occupancies *in vivo* for given steady state proportions of inactive complexes. 5. The proportion of inactive complex was related linearly to occupancy of site 1. 6. Sodium dithionite (10 mM) and Ca^{2+} (0.5 μM) together resulted in faster dephosphorylations of each site than either agent alone; relative rates were site 2 > (1 = 3). 7. Dephosphorylation and possibly phosphorylation of sites 1 and 2 was not purely sequential as shown by detection of complexes phosphorylated in site 2 but not in site 1. Estimates of the contribution of site 2 phosphorylation to inactivation ranged from 0.7 to 6.4%. 8. It is concluded that the primary function of site 1 phosphorylation is inactivation, phosphorylation of site 2 is not primarily concerned with inactivation and that phosphorylation of site 3 is non-inactivating.

Mammalian pyruvate dehydrogenase complexes (EC 1.2.4.1 + EC 2.3.1.12 + EC 1.6.4.3) are phosphorylated and inactivated by an intrinsic kinase (EC 2.7.1.99) and dephosphorylated and reactivated by a phosphatase (EC 3.1.3.43) (Linn *et al.*, 1969). Three phosphorylation sites are found in purified complexes (for reviews see Reed *et al.*, 1980; Randle *et al.*, 1981) and also in complexes in rat heart mitochondria, in rat heart *in vivo* and in rat adipocytes, which suggests they are physiologically important (Sale & Randle, 1980, 1981a; Hughes *et al.*, 1980).

Investigations into the precise biological roles of the three phosphorylation sites have been hindered by lack of a practical method of recovering each site

in a separate peptide and thus of unambiguously assaying site occupancies. Sale & Randle (1981b) have recently overcome this problem for pig heart complex by combining established methods of tryptic digestion (Davis *et al.*, 1977) with formic acid cleavage. Trypsin yields a tetradecapeptide (TA) containing sites 1 (Ser-5) and 2 (Ser-12) [sequence 2 of Sugden *et al.* (1979) for pig heart complex] and a nonapeptide (TB) containing site 3 [sequence 1 of Sugden *et al.* (1979)] (see also the legend to Table 1). Three TA [^{32}P]phosphopeptides may be produced, TA1 (^{32}P at Ser-5), TA2 (^{32}P at Ser-12) and TA12; TA1 and TA2 coelectrophorese. Formic acid quantitatively cleaves pig heart TA peptides at Asp⁸–Pro⁹, located between sites 1 and 2. The resulting phosphopeptides, TF1 (residues 1–8, site 1) and TF2 (residues 9–14, site 2) may be separated by electrophoresis (pH 2) thus allowing ^{32}P to be

Abbreviation used: ATP γ S, adenosine 5'-[γ -thio]triphosphate.

assigned unequivocally to sites 1 and 2. It is shown here that the method is applicable to the rat heart complex.

Application of trypsin/formic acid cleavage to analysis of phosphorylation and inactivation in purified pig heart complexes showed that relative initial rates of phosphorylation were site 1 > site 2 > site 3, and that site 1 was the predominant inactivating site with the contribution of site 2 being about 1.6% over the range 1.5–90% of complex inactivated (Sale & Randle, 1981b). The method is applied here to inactivation of complex in intact mitochondria.

Relative rates of dephosphorylation of fully phosphorylated purified complexes is site 2 > site 1 or 3. Based on trypsin/thermolysin cleavage (which lacks precision: see Sale & Randle, 1981b), site 1 may be the major inactivating site during dephosphorylation (Kerbey *et al.*, 1981). It is necessary to ascertain whether complex in mitochondria behaves similarly to purified complexes. In the present paper trypsin/formic acid cleavage has been applied to complex in intact rat heart mitochondria to study dephosphorylation and steady state site occupancies and to define roles of individual sites in maintaining inactive complex under these conditions.

In mitochondria, kinase and phosphatase are simultaneously active (Randle *et al.*, 1978) and their relative activities towards sites 1/2/3 determine site occupancies and the proportion of inactive complex. In this study dephosphorylation has been induced by inhibiting the kinase reaction, or followed in the steady state by turnover (i.e. by substitution of ^{31}P for ^{32}P), or with Ca^{2+} which activates the phosphatase (Denton *et al.*, 1972; Severson *et al.*, 1974). Ca^{2+} is also an inhibitor of the kinase reaction but only at low $[\text{Mg}^{2+}]$ (Cooper *et al.*, 1974). Extra-mitochondrial Ca^{2+} induces conversion of inactive complex into active complex in rat heart mitochondria (Hansford & Cohen, 1978; Denton *et al.*, 1980). This is shown to be due to dephosphorylation.

Experimental

Materials

Sources of biochemicals, chemicals, radiochemicals, enzymes and rats were as in Sale & Randle (1980, 1981a,b). Rat heart pyruvate dehydrogenase complex was obtained by dephosphorylation of partially purified pyruvate dehydrogenase phosphate complex (Sale & Randle, 1981a).

Methods

Preparation of rat heart mitochondria (Nagarse method: normal fed rats) mitochondrial incubations, measurements of mitochondrial protein-bound ^{32}P ,

of ^{32}P in tryptic phosphopeptides, and of active complex were as in Sale & Randle (1980) apart from differences given below. In some experiments mitochondria were prepared without Nagarse proteolysis (see Kerbey *et al.*, 1976); mitochondria prepared by the two methods gave similar results. Precision in measurement of the time course of phosphorylation/dephosphorylation of individual sites was much improved by incubations in bulk from which 250 μl samples were removed at prescribed time intervals into 60 μl of 7% (v/v) Triton-X-100/150 mM-EDTA/75 mM-NaF at 0°C. Samples for analysis of protein-bound ^{32}P ($4 \times 20 \mu\text{l}$) were then taken at leisure at the end of the experiment. The remainder of each sample was processed for tryptic digestion by precipitation [10% (v/v) trichloroacetic acid], washing with methanol (to remove Triton) and then two washes with 10% (v/v) trichloroacetic acid. Control experiments showed no significant differences in total ^{32}P incorporation or site occupancies between this method and the original method of Sale & Randle (1980). Monosodium monopotassium 2-oxoglutarate replaced the dipotassium salt used by Sale & Randle (1980).

Tryptic and tryptic/formic acid phosphopeptides were prepared, separated by high voltage paper electrophoresis and eluted as described by Sale & Randle (1981b). In preparing tryptic peptides performic acid oxidation of mitochondrial proteins was omitted (cf. Sale & Randle, 1980).

Free $[\text{Ca}^{2+}]$ at pH 7.2 (measured at 30°C) were computed by the methods given in Severson *et al.* (1974) and Portzehl *et al.* (1964).

In the present study ^{32}P incorporations into pyruvate dehydrogenase complex in rat heart mitochondria incubated for 10 min with 5 mM-2-oxoglutarate/0.5 mM-L-malate averaged 181 ± 7 pmol of P/mg of mitochondrial protein and 2.39 ± 0.1 nmol/unit of complex inactivated (mean $\pm$ s.e.m. for 64 observations). These values are similar to those obtained previously (Sale & Randle, 1980).

Results and discussion

Measurement of phosphorylation site occupancies in rat heart complex by trypsin/formic acid cleavage.

Rat heart pyruvate dehydrogenase [^{32}P]phosphate complexes yield tryptic [^{32}P]phosphopeptides on electrophoresis at pH 2 indistinguishable from those of pig heart complex (Sale & Randle, 1980; for R_F values and definitions see Table 1). Tryptic/formic acid [^{32}P]phosphopeptides were likewise indistinguishable (for R_F values and definitions see Tables 1 and 2). Phosphopeptide TA12 (rat or pig heart) gave with formic acid near-equal amounts of two phosphopeptides TF1 and TF2 (ratio TF1/TF2, 0.94–0.99). Pig heart complex TF1 contains ^{32}P in

Table 1. Mobilities relative to *N*⁶-dinitrophenyl-lysine (R_F) of tryptic and tryptic/formic phosphopeptides from purified pig and rat heart pyruvate dehydrogenase [³²P]-phosphate complexes on high voltage paper electrophoresis at pH 2

R_F values are given as means $\pm$ S.E.M. for the number of observations in parentheses. Tryptic phosphopeptides are denoted by T; tryptic/formic acid phosphopeptides by TF. For pig heart, TA peptides are (Tyr-His-Gly-His-Ser-Met-Ser-Asp-Pro-Gly-Val-Ser-Tyr-Arg) and TB peptide is (Tyr-Gly-Met-Gly-Thr-Ser-Val-Glu-Arg). TA1(A2) is a mixture of TA1 [TA phosphorylated at site 2 (Ser-5)] and TA2 [TA phosphorylated at site 2 (Ser-12)]. TA12 is TA phosphorylated at sites 1 and 2. TB3 is TB phosphorylated at site 3 (Ser-6) (Sugden *et al.*, 1979). TF1 is residues 1–8 of TA1 or TA12 and TF2 is residues 9–14 of TA2 or TA12 formed by formic acid cleavage of Asp₈-Pro₉. TF3 is the major phosphopeptide after formic acid treatment of TB3 and is assumed to be unchanged TB3 (Sale & Randle, 1981b). The amino acid sequence or composition of the equivalent rat heart phosphopeptides is not known. For further details see the legend to Table 2 and the Experimental section.

Phosphopeptide	R_F	
	Pig heart	Rat heart
TA1(A2)	1.39 $\pm$ 0.01 (10)	1.37 $\pm$ 0.004 (54)
TA12	1.01 $\pm$ 0.01 (11)	1.00 $\pm$ 0.003 (54)
TB3	0.72 $\pm$ 0.02 (11)	0.71 $\pm$ 0.004 (54)
TF1	1.34 $\pm$ 0.01 (24)	1.35 $\pm$ 0.005 (111)
TF2	0.88 $\pm$ 0.01 (8)	0.88 $\pm$ 0.003 (108)
TF3	0.74 $\pm$ 0.01 (10)	0.73 $\pm$ 0.003 (46)

site 1 and TF2 contains ³²P in site 2 (shown by purification and amino-acid analysis; Sale & Randle, 1981b). The identities of TF1 and TF2 from rat heart complex TA12 are assumed from their electrophoretic mobilities (Tables 1 and 2; lack of material precluded characterization by amino-acid analysis and sequence determination). TA1(A2) from either source was largely unchanged by formic acid, as expected if TF1 was the major product [mobility of TF1 is closely similar to that of TA1(A2), Table 1]. A lesser peak from rat and pig heart TA1(A2) (R_F 0.88) and variable in amount (up to 14%) depending upon the source of TA1(A2) (see later) was also formed. It is assumed to represent TF2 formed from TA2 in TA1(A2). This peak was essentially absent from TF1 re-treated with formic acid (Table 2). Formic acid treatment of TA1(A2), TA12, TB3, TF1, TF2 and TF3 from pig heart complex also gives rise to minor uncharacterised products (Sale & Randle, 1981b). Data in Table 2 [(a), (b) and (c)] show that these minor products have the same R_F and are formed in equivalent proportions with both rat and pig heart complexes. This might suggest closely similar amino-acid sequences of TA and TB in rat and pig.

Fig. 1. Relationship between inactivation and incorporation of ³²P_i into sites 1 (●), 2 (□) and 3 (▲) of pyruvate dehydrogenase complex in rat heart mitochondria

Rat heart mitochondria (2 mg of protein/ml) were preincubated for 10 min at 30°C in KCl medium (0.2 mM-³²P_i, 5.5 d.p.m./fmol, no substrate) to effect dephosphorylation of pyruvate dehydrogenase phosphate. Monosodium 2-oxoglutarate + potassium L-malate (adjusted to pH 7.4 at 23°C with KOH) were added to 5 mM and 0.5 mM respectively and samples were taken during incubation for the times shown on the right-hand side. Site occupancies were determined by tryptic digestion alone [assuming that all TA1(A2) is TA1] (solid lines; ●, □) and by cleavage of TA1(A2) with formic acid to ascertain true occupancies of sites 1 and 2 (broken lines). Activities were determined in parallel non-radioactive incubations. Each point is the mean for two experiments and eight observations.

Site occupancies in rat heart complex were calculated as follows. The ³²P contents of TA1(A2), TA12, and TB3 were determined; ³²P in TB3 was assigned to site 3 and ³²P in TA12 was assigned 0.5/0.5 to sites 1 and 2 unless both ³²P and ³¹P were present (as in the turnover experiments). The ³²P contents of sites 1 and 2 in TA1(A2) and TA12 in turnover experiments were determined by formic acid cleavage after isolation by electrophoresis and elution. In those experiments in which tryptic digestion alone was performed, TA1(A2) was assumed to be solely TA1 (see Sale & Randle, 1980).

Phosphorylation site occupancies during inactivation of pyruvate dehydrogenase complex in rat heart mitochondria

In the experiments shown in Figs. 1 and 2(a) mitochondria were pre-incubated with ³²P_i but

Table 2. ^{32}P -distribution (%) upon cleavage of tryptic phosphopeptides from rat heart and pig heart pyruvate dehydrogenase [^{32}P]phosphorylated complexes with formic acid

Phosphopeptides were isolated from tryptic digests by high-voltage paper electrophoresis (pH 2) and elution by descending chromatography. Incubations with formic acid (90%, v/v) were for 140–150 h at 37°C, then ^{32}P distributions were determined by electrophoresis at pH 2. R_F values are mobilities relative to N^6 -dinitrophenyl-lysine. For definition of TA12, TA1(A2), TB3, TF1, TF2 and TF3 see the legend to Table 1; (a), (b) and (c) are uncharacterized ^{32}P compounds generated by formic acid treatment. Retreatments with formic acid (lines 9–12) on TF1 and TF2 (and TF3; Sale & Randle, 1981b) showed that (a) is formed from TF1, (b) is formed from TF3 and (c) is products formed from TF2 or TF3. TA12 and TB3 were from fully phosphorylated complexes. TA1(A2) was from partially phosphorylated complexes formed during incremental phosphorylation with [γ - ^{32}P]ATP (purified complexes) or by incubation of mitochondria (preincubated without substrate) with $^{32}\text{P}_i$ /2-oxoglutarate/L-malate (see Fig. 1). Values are means $\pm$ S.E.M.

Phosphopeptide and source	n	% of total ^{32}P in:						
		TF1	TF2	TA12	TF3	(a)	(b)	(c)
TA12, pig (purified)	3	46 $\pm$ 0.6	48 $\pm$ 1.2	3 $\pm$ 0.7	—	2 $\pm$ 0.5	—	1 $\pm$ 0.3
TA12, rat (purified)	5	45 $\pm$ 1.1	48 $\pm$ 1.6	3 $\pm$ 0.6	—	2 $\pm$ 0.4	—	2 $\pm$ 0.1
TA12, rat (mitochondria)	50	46 $\pm$ 0.2	48 $\pm$ 0.2	2 $\pm$ 0.5	—	2 $\pm$ 0.1	—	2 $\pm$ 0.1
TA1(A2), pig (purified)	8	95 $\pm$ 0.3	2 $\pm$ 0.2	—	—	3 $\pm$ 0.2	—	—
TA1(A2), rat (purified)	1	93	<3	—	—	4	—	—
TA1(A2), rat (mitochondria)	7	92 $\pm$ 0.3	3 $\pm$ 0.2	—	—	5 $\pm$ 0.1	—	—
TB3, pig (purified)	5	—	—	—	84 $\pm$ 0.5	—	12 $\pm$ 0.5	4 $\pm$ 0.4
TB3, rat (purified)	7	—	—	—	81 $\pm$ 0.4	—	14 $\pm$ 0.5	5 $\pm$ 0.3
TF1, pig (purified)	8	96 $\pm$ 0.2	<0.3	—	—	4 $\pm$ 0.2	—	—
TF1, rat (mitochondria)	7	96 $\pm$ 0.2	<0.3	—	—	4 $\pm$ 0.1	—	—
TF2, pig (purified)	1	—	97	—	—	—	—	3
TF2, rat (mitochondria)	1	—	97	—	—	—	—	3
Approximate R_F electrophoresis pH 2	—	1.35	0.88	1.0	0.72	0.72	1.29	0.18

Fig. 2. Relative concentrations of [^{32}P]phosphopeptides TA1 (■), TA2 (▲) and TA12 (□) in pyruvate dehydrogenase [^{32}P]phosphate complexes in rat heart mitochondria

Values for TA12 ($0.5 \times ^{32}\text{P}$) and TA1(A2) were obtained by tryptic digestion, and values for TA1 and TA2 in TA1(A2) were then obtained by formic acid cleavage of TA1(A2). (a) Experimental details as in Fig. 1; (b) experimental details as in Fig. 3; (c) experimental details as in Fig. 4.

without respiratory substrate for 10 min at 30°C to effect dephosphorylation and reactivation of the complex. Phosphorylation *de novo* and inactivation was then induced by addition of 2-oxoglutarate (to

5 mM) and L-malate (to 0.5 mM). Details regarding assumptions made and their justification are given in Sale & Randle (1980). Fig. 1 shows the relationship between incorporation of ^{32}P into sites 1, 2 and 3 (as

% maximum total incorporation) and inactivation of the complex (as % total complex). The solid line shows site occupancies based on tryptic analysis and assuming all TA1(A2) is TA1; the dashed lines show true occupancies based on tryptic analysis of TA12, TA1(A2), and TB3 followed by formic acid analysis of TA1(A2). Control experiments confirmed that TA12 gave equal amounts of TF1 and TF2 after formic acid treatment (ratio TF1/TF2, 0.99 ± 0.02 ; mean $\pm$ s.e.m. for seven observations). The results in Fig. 1 show unequivocally that relative initial rates of phosphorylation were site 1 > site 2 > site 3 and that phosphorylation of site 1 was linearly correlated with inactivation of the complex (slope 2.15 ± 0.05 ; intercept 0.31 ± 1.3 ; $r = 1.0$). This correlation was not affected by the inclusion of the contribution of complexes forming TA2.

In Fig. 1 the difference between broken and solid lines represents the potential contribution of site 2 to inactivation because it represents pyruvate dehydrogenase α -chains phosphorylated in site 2 but not site 1. The potential contributions of sites 1 and 2 to inactivation is shown in more detail in Fig. 2(a), which gives the time course of appearance of individual TA phosphopeptides. The data show that the course of events is principally phosphorylation of site 1 (i.e. TA1) followed by phosphorylation of site 2 (i.e. TA12), and that phosphorylation of site 1 is principally responsible for inactivation. Fig. 2(a) also shows that phosphorylation of site 2 may precede that of site 1 (i.e. TA2) and may potentially make a small but significant ($P < 0.001$ for difference from zero) contribution to inactivation; this contribution was 1–2.5%. This conclusion assumes that phosphorylation of site 2 is inactivating; what has been shown experimentally is that thiophosphorylation of site 2 and site 3 is inactivating (Tonks *et al.*, 1982) (see later discussion).

Experiments were also performed omitting pre-incubation of mitochondria which involve both phosphorylation *de novo* and turnover of inactive complex (see Sale & Randle, 1980). Cleavage of TA12 with formic acid showed at all time points a ratio TF1/TF2 of 0.92 ± 0.02 (mean $\pm$ s.e.m. for six observations). The relative rates of phosphorylation were sites 1 > 2 > 3 and incorporation of ^{32}P into site 1 was linearly correlated with inactivation (results not shown). The potential contribution of phosphorylation of site 2 to inactivation (i.e. TA2) was 0.7–2.2% (results not shown).

Dephosphorylation of fully phosphorylated pyruvate dehydrogenase [^{32}P]phosphate in rat heart mitochondria

In experiments described below maximum incorporation of ^{32}P into all sites of phosphorylation in the complex was achieved by pre-incubating freshly prepared mitochondria with 5 mM-2-oxoglutarate/

0.5 mM-L-malate/0.2 mM- $^{32}\text{P}_i$ (1–7 d.p.m./fmol) for 10 min at 30°C (see Sale & Randle, 1980). At this steady state the characteristics of pyruvate dehydrogenase complex were (mean $\pm$ s.e.m., 14 experiments): inactive complex, $93.5 \pm 0.76\%$ of total complex; relative TA phosphopeptide concentrations, TA1, $16.5 \pm 1.8\%$, TA2 $0.73 \pm 0.04\%$, TA12 $82.8 \pm 1.9\%$; ^{32}P (% of total ^{32}P): site 1, 36.1 ± 0.5 ; site 2, 31.7 ± 0.7 ; site 3, 32.3 ± 0.5 . Formic acid cleavage of TA12 gave a ratio (TF1/TF2) of 0.93 ± 0.02 (mean $\pm$ s.e.m., eight experiments). It is concluded that turnover and phosphorylation *de novo* with ^{32}P were substantially complete. At the end of the period of pre-incubation the pyruvate dehydrogenase kinase reaction was inhibited with (a) potassium dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase (Whitehouse *et al.*, 1974) or (b) sodium dithionite (to remove O_2 and thus inhibit respiration and substrate level and respiratory chain phosphorylations) or (c) sodium pyruvate, an inhibitor of pyruvate dehydrogenase kinase (Linn *et al.*, 1969) or (carbonyl cyanide *m*-chlorophenylhydrazine + oligomycin + sodium arsenite) which collectively inhibit respiratory chain phosphorylation and 2-oxoglutarate dehydrogenase. Under these conditions the phosphatase reaction predominated in mitochondria and reactivation of the complex and dephosphorylation of individual sites was followed as given below.

(a) *Dephosphorylation and reactivation with 1 mM-potassium dichloroacetate.* As shown in Fig. 3, addition of potassium dichloroacetate caused rapid dephosphorylation and reactivation of the complex in rat heart mitochondria (in 5 min, 51.3% reactivation and 56.5% dephosphorylation). Dephosphorylation of sites 1 and 3 was apparently first order for not less than 7 min. Plots of $\ln(\text{site 2 occupancy})$ against time were linear for 3 min but deviated from linearity thereafter (plots not given). This deviation from linearity for site 2 was seen with all methods of reactivation. Deviations from linearity may be expected as a new steady state is approached where rates of dephosphorylation and phosphorylation again become equal. The inhibitors used to induce dephosphorylation in this study are unlikely to lead to total inhibition of phosphorylation. The apparent first-order rate constants (based in the case of site 2 for values between 0 and 3 min) are given in Table 3. The relative rates of dephosphorylation were site 2 > (site 1 = site 3). With sodium dichloroacetate + 10 mM-NaCl rate constants were not significantly different (results not shown). This suggests that Na^+ salts do not exert any effects on dephosphorylations, although 5 mM- Na^+ as monosodium 2-oxoglutarate was always present.

Reactivation of the complex was correlated linearly with dephosphorylation of site 1 or site 3; slopes were (mean $\pm$ s.e.m.) 2.55 ± 0.9 (site 1) and

Fig. 3. Reactivation and dephosphorylation of sites 1 (■), 2 (□) and 3 (▲) of pyruvate dehydrogenase [^{32}P]-phosphate in rat heart mitochondria induced by 1 mM-potassium dichloroacetate

Rat heart mitochondria (2 mg of protein/ml) were preincubated for 10 min at 30°C in KCl medium containing 0.2 mM- $^{32}\text{P}_i$ (1–5.5 d.p.m./fmol) and 5 mM-monosodium 2-oxoglutarate/0.5 mM-L-malate. Potassium dichloroacetate was then added to 1 mM and samples taken for analysis at the times shown on the right-hand side. Site occupancies were determined by tryptic digestion alone (solid lines; ■, □) and by cleavage of TA1(A2) with formic acid to ascertain true occupancies of sites 1 and 2 (broken lines). Activities were determined in parallel non-radioactive incubations. Each point is the mean for 32 observations on eight preparations of mitochondria.

2.51 ± 0.19 (site 3), intercepts were 0.28 ± 2.35 and 5.0 ± 4.4 , and correlation coefficients 1.0 and 0.98. These values are based on true occupancy of site 1 obtained by tryptic cleavage followed by formic acid cleavage of TA1(A2) (broken line in Fig. 3). The values obtained with tryptic cleavage alone, which includes the contribution to inactivation of complexes phosphorylated in site 2 but not site 1, were 2.48 ± 0.11 (slope), 0.29 ± 2.76 (intercept) and 0.99 (r). These were not significantly different from those obtained by trypsin/formic acid. The contribution of site 1 and site 2 phosphorylations to the maintenance of inactive complex during dephosphorylation is shown in Fig. 2(b). Throughout, inactivation was largely maintained (>96%) by complexes phosphorylated in site 1 (i.e. yielding TA1 or TA12). The potential contribution of complexes phosphorylated in site 2 but not site 1 rose from 0.9% (zero time) to a maximum of 3.4% (1 min). No conclusions about the role of site 3 phosphorylation in maintaining inactive complex can be drawn from this experiment.

(b) *Dephosphorylation and reactivation with 10 mM-sodium dithionite.* Reaction of sodium dithionite with O_2 forms protons; control experiments showed that the pH of the incubation remained above 7 for at least 10 min. Addition of dithionite led to dephosphorylation and reactivation of complex in rat heart mitochondria as shown in Fig. 4 (in 5 min, 47% reactivation and 49% dephosphorylation). In this Figure, as in Figs. 1 and 3, true occupancies are denoted by broken lines and approximate occupancies (based on tryptic analysis alone) by solid lines. With dithionite, relative rates of dephosphorylation were sites $2 > 1 > 3$, as shown in Fig. 4 or by the apparent first-order rate constants in Table 3. With dithionite (as compared with dichloroacetate) rates of dephosphorylation were slower, and dephosphorylation of site 3 was slower than that of site 1 (Table 3).

Table 3. Apparent first-order rate constants for dephosphorylation of pyruvate dehydrogenase [^{32}P]phosphate in rat heart mitochondria

Experimental details were as in the legends to Figs. 1, 3 and 4. For differences between values for sites 1, 2, 3 on any line $P < 0.001$ except where marked * ($P > 0.05$ for site 1 versus site 3). Analyses by trypsin/formic acid except † (trypsin only). Rate constants are based on mean values for at least five different time points. CCCP is carbonyl cyanide *m*-chlorophenylhydrazone. Rate constants are means $\pm$ S.E.M.

Experiment	Rate constant (min^{-1})		
	Site 1	Site 2	Site 3
Potassium dichloroacetate (1 mM)	$-0.13 \pm 0.004^*$	-0.34 ± 0.004	-0.13 ± 0.004
	† $-0.13 \pm 0.003^*$	-0.37 ± 0.006	-0.13 ± 0.004
Sodium dithionite (10 mM)	-0.11 ± 0.11	-0.28 ± 0.021	-0.05 ± 0.003
	† -0.11 ± 0.007	-0.32 ± 0.023	-0.05 ± 0.003
Sodium pyruvate (5 mM)	-0.09 ± 0.006	-0.15 ± 0.023	-0.05 ± 0.007
	† -0.07 ± 0.004	-0.23 ± 0.01	-0.03 ± 0.007
CCCP (1 μM), oligomycin (40 $\mu\text{g}/\text{ml}$), sodium arsenite (10 mM) $^{31}\text{P}_i$ (5 mM) (turnover)	-0.07 ± 0.006	-0.18 ± 0.007	-0.02 ± 0.003
	$-0.43 \pm 0.07^*$	-0.53 ± 0.09	-0.38 ± 0.05
Sodium dithionite (10 mM) and Ca^{2+} (0.5 μM)	† $-0.40 \pm 0.06^*$	-0.57 ± 0.11	-0.38 ± 0.05

Fig. 4. Reactivation and dephosphorylation of sites 1 (■), 2 (□) and 3 (▲) of pyruvate dehydrogenase [^{32}P]phosphate in rat heart mitochondria induced by 10 mM-sodium dithionite

Details were as given in the legend to Fig. 3, except that 10 mM-sodium dithionite was used in place of 1 mM-potassium dichloroacetate. Values are mean for 16 observations on four preparations of mitochondria.

Reactivation of the complex was correlated linearly with dephosphorylation of site 1 as estimated either by tryptic/formic acid cleavage (broken line in Fig. 4) or by tryptic cleavage alone (solid line in Fig. 4) (tryptic cleavage alone underestimates dephosphorylation of site 1 because TA2 is assumed to be TA1). Values (mean $\pm$ S.E.M.) were: slopes 2.65 ± 0.15 , 2.55 ± 0.3 ; intercepts, 4.38 ± 3.2 , 4.46 ± 2.96 ; $r = 0.99$, 0.99 , respectively. Reactivation of the complex appeared more rapid than dephosphorylation of site 3; thus at 50% reactivation, only 25% of site 3 was dephosphorylated. This may show that phosphorylation of site 3 is not inactivating. As shown in Fig. 2(c), complexes phosphorylated in site 2 but not site 1 (TA2) were formed with dithionite. Potentially their contribution to inactivation was as high as 6–7%. Nevertheless, complexes phosphorylated in site 1 (TA1, TA12) were the major inactivating species.

Relative rates of dephosphorylation of sites 1, 2 and 3 in mitochondria of alloxan-diabetic rats showed no obvious differences from those of non-diabetic rats (results not given).

(c) *Dephosphorylation with 5 mM-sodium pyruvate or with (1 μM -carbonyl cyanide *m*-chloro-*

phenylhydrazine + 40 μg of oligomycin/ml + 10 mM-sodium arsenite). In experiments with these inhibitors site occupancies were measured by tryptic digestion alone and assuming that all TA1(A2) is TA1. The apparent first-order rate constants for dephosphorylation of sites 1, 2 and 3 given in Table 3 show that relative rates were sites $2 > 1 > 3$.

Turnover of ^{32}P (^{31}P replacing ^{32}P) in fully phosphorylated complex in rat heart mitochondria

Freshly prepared rat heart mitochondria incubated with 2-oxoglutarate/L-malate are at steady state from 10–40 min with respect to proportion of inactive complex and phosphorylations (Sale & Randle, 1980) and phosphate in the complex turns over (Randle *et al.*, 1978). This turnover therefore depends on the activity of the phosphatase and affords a means of investigating dephosphorylations without use of inhibitors of the kinase. This has now been realised by addition of a 25-fold excess of $^{31}\text{P}_i$ after 10 min of incubation with $^{32}\text{P}_i$; the proportion of inactive complex remained constant at 93.5%. The apparent first-order rate constants for loss of ^{32}P from each site are given in Table 3 (trypsin/formic acid analyses). The relative rates were sites $2 > 1 > 3$; the absolute values were lower than with dichloroacetate or dithionite but comparable with those seen with the other inhibitors of the kinase reaction.

Effect of extramitochondrial $[\text{Ca}^{2+}]$ on complex activity and phosphorylation site occupancies in rat heart mitochondria

Pyruvate dehydrogenase phosphate phosphatase is activated by Ca^{2+} and extramitochondrial Ca^{2+} increases the proportion of active complex in rat heart mitochondria with $K_{0.5}$ of approx. $0.5 \mu\text{M}$ (Denton *et al.*, 1972, 1980; Hansford & Cohen, 1978). As shown in Fig. 5, the proportion of inactive complex and the occupancy of phosphorylation sites in the complex in rat heart mitochondria oxidizing 2-oxoglutarate/L-malate was decreased by inclusion of Ca^{2+} (concentration range 0– $2.6 \mu\text{M}$). Occupancy of site 1 and the proportion of inactive complex were linearly related (slope 1.45 ± 0.08 ; intercept -6.1 ± 4.7 ; r , 0.99; mean $\pm$ S.E.M.). Occupancy of site 2 and site 3 decreased relative to that of site 1 with increasing Ca^{2+} ($3 > 2$). The results are broadly similar to those obtained when pyruvate is used to vary the proportion of inactive complex (Sale & Randle, 1980). The results with Ca^{2+} differ in two respects: analyses were by trypsin/formic acid, and there was perhaps evidence for a relative increase of site-3 occupancy especially at high $[\text{Ca}^{2+}]$. Complexes phosphorylated in site 2 but not in site 1 contributed $0.7 \pm 0.04\%$ of inactive species at all Ca^{2+} concentrations (mean $\pm$ S.E.M. for eight observations over the range of Ca^{2+} concentrations in Fig. 5). Control experiments showed that the

Fig. 5. Relationship between inactivation and incorporation of $^{32}\text{P}_i$ into sites 1 (■), 2 (□), and 3 (▲) of pyruvate dehydrogenase complex in rat heart mitochondria in the steady state

Rat heart mitochondria (2 mg of protein/ml) were incubated for 12 min at 30°C in KCl medium (pH 7.2 at 30°C) containing 0.2 mM- $^{32}\text{P}_i$ (7000 d.p.m./pmol), 5 mM-monosodium 2-oxoglutarate, 0.5 mM-L-malate and 2 mM-calcium/EGTA buffers giving the computed Ca^{2+} concentrations shown on the right-hand side. Site occupancies were measured by tryptic digestion and formic acid cleavage of TA1(A2). Values are means for four observations.

proportion of active complex and phosphorylation site occupancies were at steady state and that TA12 gave approx. equal amounts of TF1 and TF2 (results not shown).

In the experiments described above, Ca^{2+} was present throughout incubation. When mitochondria were incubated for 10 min with 2-oxoglutarate/L-malate/ $^{32}\text{P}_i$ and then exposed to $0.47\ \mu\text{M}\text{-Ca}^{2+}$, dephosphorylation was induced. Dephosphorylations were not first order but relative rates were sites $3 > 2 > 1$ (or in one experiment sites $3 = 2 > 1$) (results not shown). After 10 min of exposure to Ca^{2+} a new steady state was reached corresponding to ^{32}P losses of 24% (site 1), 55% (site 2) and 62% (site 3). Dephosphorylation induced by Ca^{2+} is assumed to result from activation of the phosphatase. It is suggested that rephosphorylation due to persistent kinase activity (sites $1 > 2 > 3$) modifies the normal pattern of dephosphorylations by phosphatase. The alternative explanation, that Ca^{2+} alters relative rates of dephosphorylation of individual sites, seems less likely; with purified pig heart complex and $43\ \mu\text{M}\text{-Ca}^{2+}$, relative rates of dephosphorylation were sites $2 > 3 = 1$ (Kerbey *et al.*,

1981). The results may establish that reactivation of complex induced by Ca^{2+} in mitochondria is due to net dephosphorylation.

When 10 mM-dithionite (to inhibit the kinase reaction) was added with Ca^{2+} ($0.5\ \mu\text{M}$), the apparent first-order rate constants for dephosphorylation (Table 3) were sites $2 > 3 = 1$. The rates were greater than with Ca^{2+} or dithionite alone. Compared with dithionite alone the lag in site 3 dephosphorylation (Fig. 4) was absent and there was less difference between rate constants for sites 1 and 2. With dithionite + Ca^{2+} the potential contribution to inactivation of complexes phosphorylated in site 2 but not site 1 rose from 0.8% (zero time) to a maximum of 4–7% (2 min) (time course not shown).

Errors in measurement by trypsin alone of site occupancies

Errors arise because the trypsin method does not detect TA2 and ^{32}P in site 2 in TA1(A2) is assigned to site 1. In the present study the error in occupancy was $< 10\%$ (site 1) or $< 15\%$ (site 2); the error in rate constants (Table 3) was $< 15\%$ (sites 1 or 2).

General discussion and conclusions

The primary purpose of this investigation was to investigate the role of individual sites of phosphorylation in inactivation of the pyruvate dehydrogenase complex in rat heart mitochondria. To this end we have developed a new method of measuring unambiguously occupancy of individual phosphorylation sites and shown here its applicability to the rat heart complex. A further purpose was to investigate rates of dephosphorylation of individual sites in fully phosphorylated complex in rat heart mitochondria; this has not been attempted previously.

During phosphorylation and inactivation of complex in rat heart mitochondria relative initial rates were shown unequivocally to be site $1 > \text{site } 2 > \text{site } 3$, as in purified pig heart complex (Sale & Randle, 1981b). Complex phosphorylated in site 2 but not in site 1 was detected for the first time, in rat heart mitochondria (this study) and pig heart complex (Sale & Randle, 1981b). This may indicate that phosphorylation of sites 1 and 2 is not strictly sequential (1 before 2). The alternative explanation, that phosphatase dephosphorylated site 1 in complexes containing TA12, seems less likely. The concentration of complex giving TA2 declined during the course of inactivation as the concentration of its potential precursor (complex giving TA12) increased. Moreover, the proportion of purified pig heart complex yielding TA2 between 1.5 and 90% inactivation is approx. 1.5% and such preparations are apparently devoid of phosphatase (Sale & Randle, 1981b). The proportion of rat heart mitochondrial complex giving TA2 was similar,

although phosphatase was present and known to be active.

Under all of the conditions tested in rat heart mitochondria in the present study the proportion of inactive complex was related linearly to the occupancy of site 1. It was not possible to establish by such plots whether complexes phosphorylated in site 2 but not site 1 were inactive because their potential contribution to inactivation was always small (<7%). This type of plot did, however, show that site 3 phosphorylation is unlikely to be inactivating (Fig. 4). Reed *et al.* (1980) have reached similar conclusions on the basis of experiments with ox kidney complex assumed to be thiophosphorylated in site 3 (this was not established with [^{35}S]ATP γS). Evidence that site 2 phosphorylation is inactivating is based upon the observation that pig heart complex thiophosphorylated in sites 2 and 3 with [^{35}S]ATP γS but with site 1 empty is inactive (Tonks *et al.*, 1982). The interpretation assumes that thiophosphorylation is equivalent to phosphorylation, and that thiophosphorylation of site 3 plays no role in inactivation. In the present paper estimates of the potential contribution of site 2 phosphorylation to inactivation assume that site 2 phosphorylation alone is inactivating. Because the contribution was always small it may be concluded that site 1 is the major site of inactivation.

With all methods used to induce release of $^{32}\text{P}_i$ from complex in mitochondria fully phosphorylated with ^{32}P , the rate of dephosphorylation of site 2 was approx. 2.5-fold that of site 1; dephosphorylations were first-order except with Ca^{2+} alone. These results are in broad agreement with those found for purified ox kidney and pig heart complexes (Teague *et al.*, 1979; Kerbey *et al.*, 1981). They may also confirm that the corrections which are necessary for the assumption that all TA1(A2) is TA1 are not large (*cf.* Kerbey *et al.*, 1981). With purified complexes (including rat heart; G. J. Sale & P. J. Randle, unpublished work) the rate of dephosphorylation of site 3 approximates to that of site 1. In rat heart mitochondria the rate of dephosphorylation of site 3 was either equal to that of site 1 (potassium dichloroacetate; sodium dichloroacetate + 10 mM-NaCl; sodium dithionite + Ca^{2+}) or less than that of site 1 (sodium dithionite; sodium pyruvate; carbonylcyanide *m*-chlorophenylhydrazine + oligomycin + sodium arsenite; turnover studies). The reason for this discrepancy in the rate of dephosphorylation of site 3 with different inhibitors is not known. The phenomenon did not correlate with the use of Na^+ salts, but may have correlated with conditions that gave slower rates of reactivation or zero reactivation (turnover experiment) (Table 3).

The formation in mitochondria during dephosphorylation of complex phosphorylated in site 2 but

not site 1 was detected for the first time (with dichloroacetate, and with dithionite $\pm \text{Ca}^{2+}$). Attempts to demonstrate this with purified complexes using less sensitive analytical methods were unsuccessful (Kerbey *et al.*, 1981). These results show that dephosphorylation of sites 1 and 2 is not ordered (2 before 1) and may be random.

Under steady-state conditions (Fig. 5) the potential contribution to inactivation of complexes phosphorylated in site 2 but not site 1 was always <2%. Site 2 is thus unlikely to make more than a 2% contribution to inactivation in the steady state *in vivo*. The results of the present study suggest that the contribution of site 2 could increase to 6–7% during transient states involving dephosphorylation and reactivation.

In model experiments, occupancy of phosphorylation sites was measured at different proportions of active complex in the steady state by varying [Ca^{2+}] (Fig. 5). If the results with Ca^{2+} are generally applicable to the situation *in vivo* then they may predict occupancies *in vivo* (sites 1/2/3) of 1/0.6/0.4 in fed normal rats (70% inactive complex) and of 1/1/1 in diabetic rats (99% inactive complex). These predicted values are closely similar to those recently obtained experimentally employing [$\gamma\text{-}^{32}\text{P}$]ATP back titration (Sale & Randle, 1981a) combined with trypsin/formic acid cleavage and improved techniques for preventing dephosphorylation of inactive complex during isolation. The values were 1/0.6/0.4 (fed normal) and 1/1/0.96 (diabetic) (G. J. Sale & P. J. Randle, unpublished work). The occupancies with Ca^{2+} in the present study with mitochondria were broadly similar to those obtained when pyruvate was used to vary the proportion of active complex (Sale & Randle, 1980) except that site 3 occupancy with Ca^{2+} was greater than with pyruvate.

It is concluded that site 1 is the major inactivating site; that site 2 is no more than a minor site of inactivation (0.7–7%); and that site 3 is not inactivating. The major function of site 2 and the only function of site 3 may be to inhibit reactivation by the phosphatase (Sugden *et al.*, 1978).

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