Multi-site phosphorylation of bovine kidney branched-chain 2-oxoacid dehydrogenase complex

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The α-subunit of the E_1 component of branched-chain 2-oxoacid dehydrogenase is phosphorylated at 3 sites by an endogenous kinase. Inactivation of the complex correlates with rapid phosphorylation of one of these sites. The similarities with the covalent regulation of pyruvate dehydrogenase complex are discussed.

Branched-chain 2-oxoacid dehydrogenase complex *Multi-site phosphorylation* *Inactivation*
Pyruvate dehydrogenase complex *(Bovine kidney cortex)*

1. INTRODUCTION

Two of the 3 mitochondrial 2-oxoacid dehydrogenase multi-enzyme complexes, namely, the pyruvate and branched-chain 2-oxoacid dehydrogenases, are regulated by covalent phosphorylation [1,2]. In contrast there is no evidence for phosphorylation of the 2-oxoglutarate dehydrogenase complex.

Phosphorylation of the pyruvate dehydrogenase complex has been studied extensively. Three sites on the α-subunit of the E_1 component are phosphorylated by a specific kinase which is tightly bound to the complex and co-purifies with it (the complex) [3,4]. Inactivation correlates with rapid phosphorylation of one site, although slower phosphorylation of a second site may also contribute to inactivation [5]. Phosphorylation of the third site apparently has no direct effect on the activity of the enzyme [1] but a role for the second and third sites in controlling reactivation by dephosphorylation has been postulated [6].

Branched-chain 2-oxoacid dehydrogenase complex is also inactivated by phosphorylation of the α-subunit of the E_1 component [7] and again phosphorylation is catalysed by a kinase which co-purifies with the complex [8–10]. We have reported that phosphorylation continues after complete inactivation of the complex, indicative of phosphorylation of additional sites not associated with inactivation of the enzyme [10]. Here, we report that 3 distinct sites on the α-subunit are phosphorylated by the kinase and that inactivation correlates closely with phosphorylation of one of these sites.

2. MATERIALS AND METHODS

Branched-chain 2-oxoacid dehydrogenase complex was purified from bovine kidney as in [10]. Bovine heart pyruvate dehydrogenase complex was purified as in [11]. Trypsin (TPCK-treated) was from Worthington and trypsin inhibitor (soybean) from Sigma.

Assay of branched-chain 2-oxoacid dehydrogenase activity and determination of protein-bound [^{32}P]phosphate was done as in [10]. After termination of phosphorylation by addition of glucose and hexokinase [10] aliquots were rapidly transferred into 0.2 M ammonium bicarbonate, using a micro-centrifuge desalting technique [12] and digested with trypsin. Digestion was terminated by lyophilisation or by addition of a 5-fold excess of trypsin inhibitor.

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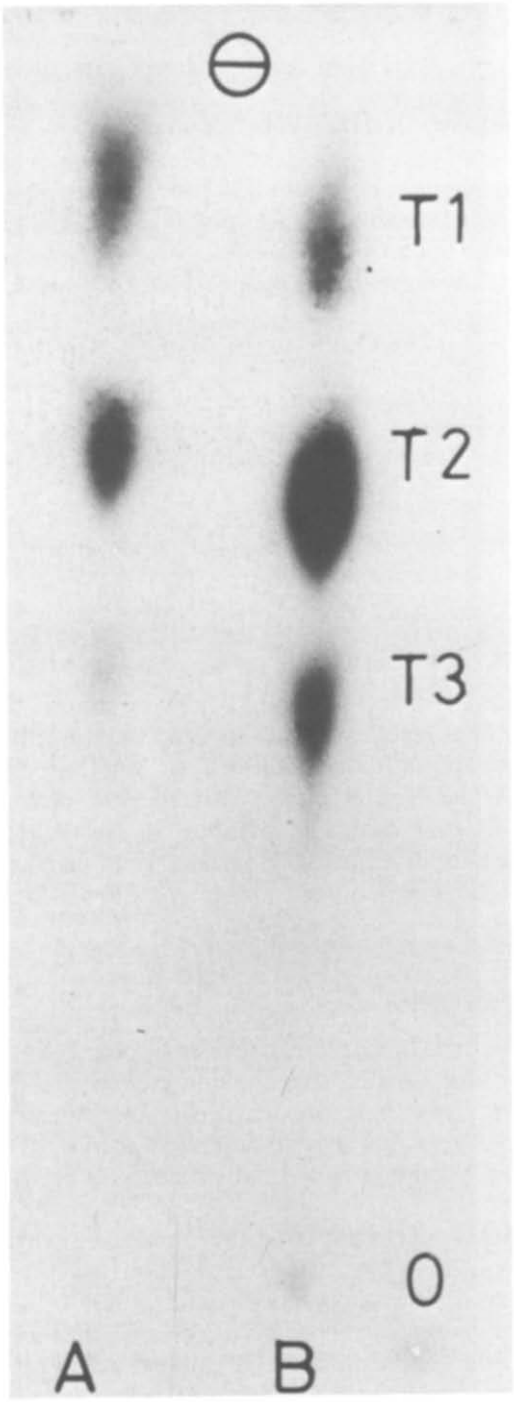

Fig.1

Fig.1. Autoradiograph of electrophoretogram (pH 1.9) of trypsin digest of: (A) branched-chain 2-oxoacid dehydrogenase complex; and (B) pyruvate dehydrogenase complex. The complexes were phosphorylated using [γ-^{32}P]ATP, transferred into 0.2 M ammonium bicarbonate, digested with trypsin (50:1, w/w) for 2 h at 20°C, lyophilised, re-dissolved in formic acid (7%, v/v) and electrophoresed at pH 1.9. The origin is denoted by O.

3. RESULTS

Pilot experiments indicated that digestion of phosphorylated branched-chain 2-oxoacid dehydrogenase complex with trypsin (50:1, w/w) yielded 3 phosphopeptides (termed T1, T2, T3) which were separable by high-voltage electrophoresis at pH 1.9 (fig.1A). Essentially all of the radioactivity was released as phosphopeptides by digestion at 20°C for 60 min. Continued digestion for up to 24 h gave the same pattern of phosphopeptides, indicating that the 3 phosphopeptides do not arise from partial cleavage at susceptible digestion sites on the enzyme. Digestion of phosphorylated pyruvate dehydrogenase complex under

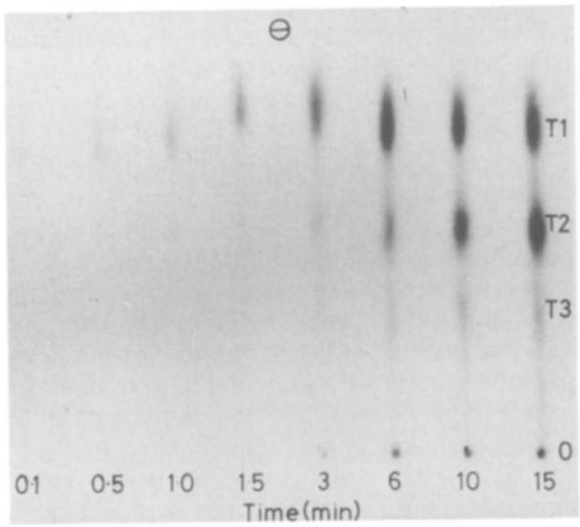

Fig.2. Time-course of phosphorylation of branched-chain 2-oxoacid dehydrogenase complex. Complex was phosphorylated for the indicated time using [γ-^{32}P]ATP. Phosphorylation was terminated by addition of glucose and hexokinase [10] and the aliquots treated as in fig.1 except that digestion was for 16 h. The origin is denoted by O.

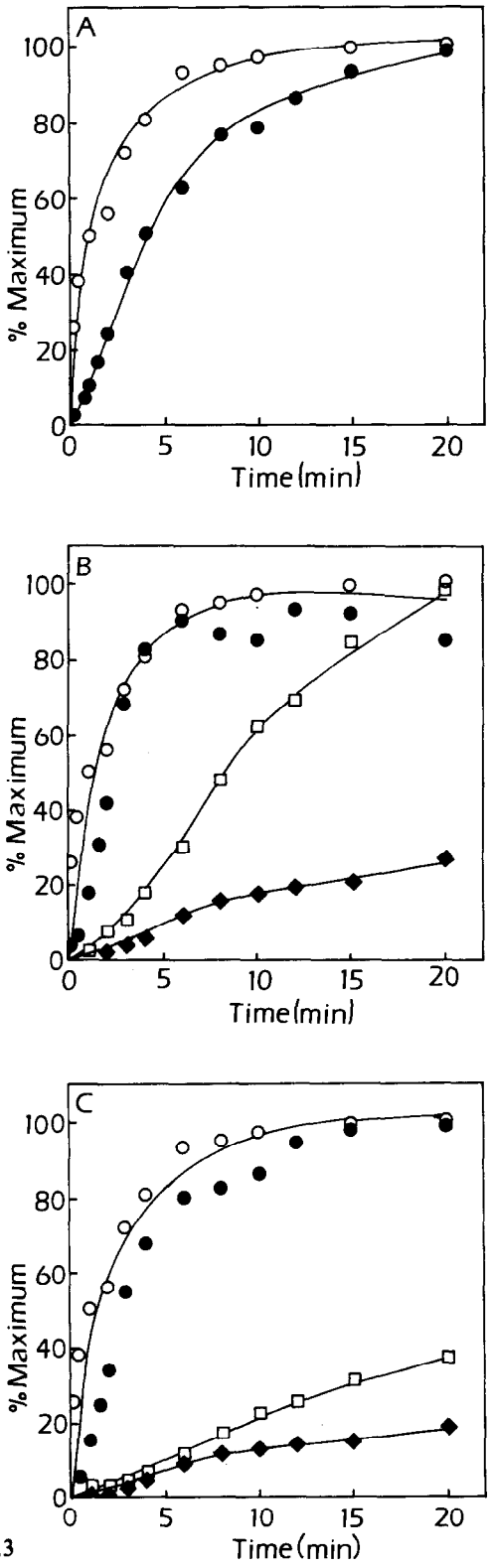

Fig.3

Fig.3. Relationship between inactivation and phosphorylation of branched-chain 2-oxoacid dehydrogenase complex. (A) Correlation of inactivation (○) with total phosphorylation (●), carried out as in [10]. (B) Correlation of inactivation (○) with phosphorylation of peptides T1 (●), T2 (□) and T3 (◆). Samples were treated as in fig.2 and radioactivity in each peptide was determined by cutting out the spot and counting in 4 ml scintillant. Values were calculated from the total phosphorylation at each time-point and expressed as a percentage of the maximum observed in any peptide. (C) Correlation of inactivation (○) with phosphorylation at sites 1 (●), 2 (□) and 3 (◆), assuming that all the radioactivity in peptide T1 is in site 1 and that half of the radioactivity in T2 is in site 1 and half in site 2. Values are expressed as a percentage of the maximum observed in any site.

identical conditions gave a very similar pattern of phosphopeptides (fig.1B).

The pattern of phosphopeptides changed with degree of phosphorylation of branched-chain 2-oxoacid dehydrogenase complex. Fig.2 shows the pattern of phosphopeptides obtained during a time-course of phosphorylation. Peptide T1 was phosphorylated most rapidly, followed by T2 and T3.

Fig.3A shows that, as found in [10], phosphorylation of branched-chain 2-oxoacid dehydrogenase continued after inactivation of the complex was almost complete. The amount of radioactivity in the 3 phosphopeptides at each time-point is shown in fig.3B. This shows clearly that inactivation of the complex correlated with phosphorylation of peptide T1.

With pyruvate dehydrogenase complex [4], peptides T1 and T2 represent the same tryptic peptide but peptide T1 is monophosphorylated and upon further slower phosphorylation of a second residue this is converted to the diphosphorylated peptide T2. In fig.3C the data for the branched-chain 2-oxoacid dehydrogenase complex are recalculated, assuming that T1 and T2 are mono- and di-phosphorylated forms of the same tryptic peptide. Again inactivation correlated with phosphorylation of the first site on T1.

4. DISCUSSION

These results indicate the presence of 3 phosphorylation sites on the α-subunit of the E_1

component of branched-chain 2-oxoacid dehydrogenase complex. Inactivation of the complex correlates with rapid phosphorylation of one of the sites. There is as yet no evidence for the possible function of phosphorylation at the second and third sites. The stoichiometry of phosphorylation cannot be calculated with any certainty as the stoichiometry of the polypeptides in the native complex is not yet known. However, if it is assumed that the polypeptides are present in a 1:1:1 ratio the maximum observed phosphorylation corresponds to ~0.6 mol phosphate/mol α-subunit, implying that: site 1 would be 40% phosphorylated; site 2, 15%; and site 3, 8%. Reasons for this low level are not clear although one possibility would be that the complex, as isolated, already contains some non-radioactive phosphate.

A striking feature of these data is the similarity between the phosphorylation of the branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase complexes [4]. Both have 3 sites of phosphorylation and inactivation correlates with rapid phosphorylation of 1 site. The phosphopeptides from the 2 complexes are released by identical digestion conditions and have similar mobility during electrophoresis. Although confirmation by sequence analysis will be necessary, 2 of the phosphorylation sites on branched-chain 2-oxoacid dehydrogenase may be closely grouped on the α-subunit, being recovered on the same tryptic peptide, as is the case with pyruvate dehydrogenase. Comparison of the sequences of the phosphopeptides from the 2 complexes and of the properties of the kinases specific to the 2 complexes will be of considerable interest.

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