Multi-site phosphorylation in ox-kidney branched-chain 2-oxoacid dehydrogenase complex

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Received 4 July 1983

Tryptic [^{32}P]phosphopeptides were prepared from [^{32}P]phosphorylated ox-kidney branched-chain complex and analysed by high-voltage paper electrophoresis at pH 1.9. In the maximally phosphorylated complex 3 tryptic [^{32}P]phosphopeptides were identified (TA, TB, TC). R_F-values relative to N^6-dinitrophenyllysine were (mean ± SEM for 25 obs.): TA, 1.53 ± 0.03; TB, 1.07 ± 0.02; TC, 0.65 ± 0.01. Relative rates of phosphorylation were TA > TB > TC. Phosphorylation of TA reached a maximum when about 66% of the complex was inactivated. Phosphorylation of TB and TC was associated mainly with 66–95% inactivation of the complex.

Branched chain 2-oxoacid dehydrogenase *Phosphorylation* *Tryptic phosphopeptide*
Phosphorylation site *Inactivation, by phosphorylation* *BCDH complex, of ox-kidney*

1. INTRODUCTION

The branched-chain 2-oxoacid dehydrogenase complex of animal tissue mitochondria is regulated by reversible phosphorylation of the α-subunit of its decarboxylase component [1–4]. Co-purification to near homogeneity of ox-kidney, rabbit liver and rat-kidney complexes together with branched-chain dehydrogenase kinase has been achieved and phosphorylation of serine residues in the α-chain of the decarboxylase component demonstrated [5–8]. Phosphorylation of purified complex is associated with inactivation, and dephosphorylation is associated with reactivation [5,8,9]. Studies of phosphorylation have been limited thus far to measurement of total incorporation of phosphate into the complex and the extent of incorporation was correlated with the degree of inactivation [5–9]. In the mitochondrial pyruvate dehydrogenase complex there are 3 sites of phosphorylation, only one of which is a major site of inactivation [10,11]. Evidence is given here (based on measurement of ^{32}P in tryptic phosphopeptides) that there are at least 3 phosphorylation sites in branched-chain dehydrogenase complex; and their occupancy has been correlated with the degree of inactivation of the complex.

Abbreviations: DTT, dithiothreitol; EGTA, ethanedioxybis (ethylamine) tetraacetate; HVPE, high-voltage paper electrophoresis

2. EXPERIMENTAL

Trypsin [treated with 1-chloro-4-phenyl-3-L-tosylaminobutan-2-one (TPCK)] was from Worthington Diagnostic Systems. Branched-chain complex was purified from ox-kidney mitochondria by the modified procedure in [8] and was free of pyruvate dehydrogenase complex by criteria given in [5,8]. Sources of other chemicals, biochemicals, X-ray film and [γ-^{32}P]ATP were as in [1,12].

Branched-chain complex activity was assayed spectrophotometrically as in [8]; one unit forms 1 μmol NADH/min at 30°C. Total incorporation of ^{32}P into the complex (from [γ-^{32}P]ATP) was measured as in [12] and corrected for blanks employing an equivalent amount of [γ-^{32}P]ATP. Sample size was such that dpm were

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00145793/83/$3.00

1300–30000/min. For tryptic digestion and analysis of tryptic phosphopeptides complex (~350 munits) was precipitated with trichloroacetic acid to 10% (w/v) and washed 6-times with 10% trichloroacetic acid to remove trichloroacetic acid-soluble ^{32}P; recovery of protein-bound ^{32}P was 60–80%. The pellet was dissolved in 25 μl 8 M urea/2% (w/v) NH_4HCO_3, the pH adjusted to 8.5–9.0 with NH_4OH (indicator paper) and diluted with 3 vol. 2% NH_4HCO_3/TPCK trypsin (1.33 mg/ml). Tryptic digestion was at 30°C for 2–4 h and monitored by disappearance of trichloroacetic acid-insoluble ^{32}P (>90%). Tryptic phosphopeptides (3250–100000 dpm in ^{32}P) were separated by HVPE at pH 1.9 and assayed for ^{32}P by liquid scintillation spectrometry as in [12] except that the distribution of ^{32}P was calculated directly from the recorded dpm. The recovery of applied ^{32}P on HVPE was 100% and the recovery in the major peaks was 81–99%. SDS–polyacrylamide gel electrophoresis in Tris–glycine and autoradiography was as in [3] except that 15 × 14 cm gels were used; about 5000 dpm in 5 munits ^{32}P-phosphorylated complex were applied. Autoradiograms were scanned on a Joyce Loebl Chromoscan 3.

Incubations of branched-chain complex with ATP were either in 30 mM potassium phosphate/5 mM DDT/5 mM EGTA/10 mM $MgCl_2$ (pH 7.50) (medium A) or in 30 mM potassium phosphate/2 mM DTT/2% (v/v) ox-serum/1 mM $MgCl_2$ (pH 7.5) (medium B). The two media gave identical patterns of ^{32}P-incorporation into tryptic phosphopeptides and identical autoradiograms of SDS gels.

3. RESULTS AND DISCUSSION

3.1. *Tryptic phosphopeptides from ^{32}P-phosphorylated ox-kidney branched-chain complex*

A scan of an autoradiogram of SDS–polyacrylamide gel electrophoresis of ^{32}P-phosphorylated branched-chain complex typical of those used in this study is shown in fig.1. The scan shows a single peak of radioactivity (M_r 49400) corresponding to the α-chain of the decarboxylase component. Phosphorylated pyruvate dehydrogenase α-chain was not detected; when

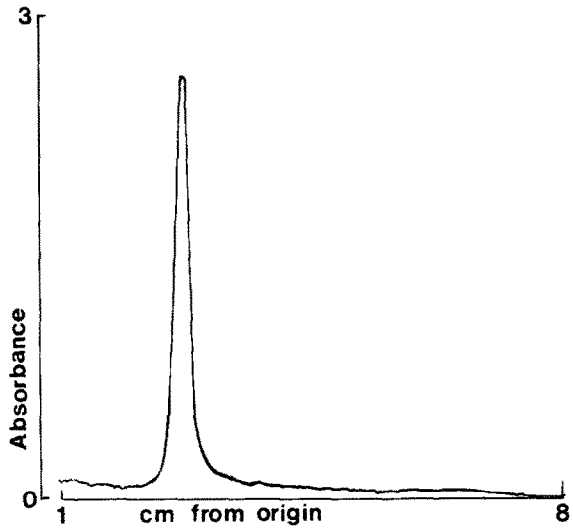

Fig.1. SDS–polyacrylamide gel electrophoresis of ^{32}P-phosphorylated ox-kidney branched-chain complex; scan of autoradiogram. Complex was incubated in medium B (see section 2) for 12 min at 30°C with 0.5 mM [γ-^{32}P]ATP (613 dpm/pmol); $t_{0.5}$ for inactivation was about 0.5 min.

present, a second peak of radioactivity is seen (M_r 41000).

Typical profiles of ^{32}P in tryptic [^{32}P]phosphopeptides obtained from ^{32}P-phosphorylated branched-chain complex by HVPE at pH 1.9 are shown in fig.2. When complex was maximally inactivated (>95%) and phosphorylated by ATP (about 2.8 nmol P/unit of complex inactivated) 3 tryptic [^{32}P]phosphopeptides were detected. The results shown are typical of 25 expt on 7 different preparations of ox-kidney branched-chain complex between January 1982 and June 1983. The R_F-values, relative to N^6-dinitrophenyllysine marker, were (mean ± SEM for 25 obs.): TA, 1.53 ± 0.03; TB, 1.07 ± 0.02; TC, 0.65 ± 0.01. In 2 of the 7 preparations a shoulder was present on the trailing edge of peak TA at 80–95% inactivation (see fig.2B) which may represent a fourth minor peak. Here, it has been assumed to be part of TA.

3.2. *Relationship between degree of inactivation and incorporation of ^{32}P from [γ-^{32}P]ATP into tryptic phosphopeptides*

Two techniques have been used to investigate the quantitative relationship between inactivation and

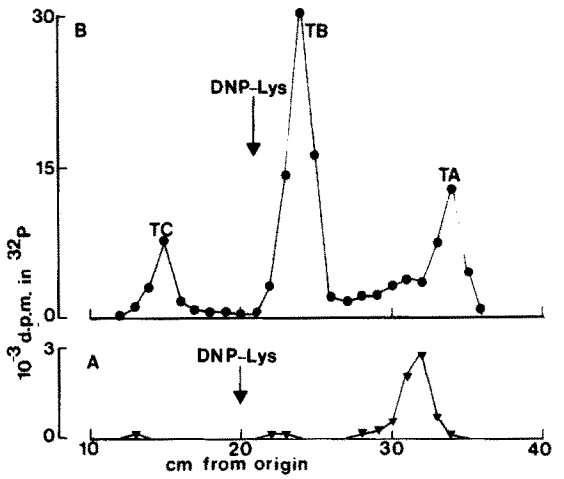

Fig.2. HVPE at pH 1.9 of tryptic digest of ^{32}P-phosphorylated branched-chain complex. Profiles were obtained from complex incubated in medium B (see section 2) for 12 min at 30°C with either: (A) 0.25 μM [γ-^{32}P]ATP (613 dpm/pmol, 4.6% inactivation); or (B) 0.5 mM [γ-^{32}P]ATP (613 dpm/pmol, 94% inactivation). The profiles are typical of those seen in 25 expt with 7 different preparations of branched-chain complex.

the profile of tryptic [^{32}P]phosphopeptides from phosphorylated branched-chain complex. With two preparations which exhibited slow inactivation by ATP ($t_{0.5}$ ~7 min) complex was incubated with 0.5 mM ATP and samples taken at suitable time intervals for assay of complex activity and incorporation of ^{32}P into tryptic phosphopeptides (fig.4(b)). This technique is not readily applicable to preparations of complex which are rapidly inactivated by ATP ($t_{0.5}$ ~0.5 min) because of the difficulty of taking multiple samples at precise times. A more convenient technique is based upon that used for pyruvate dehydrogenase phosphorylation in [12,13]. In this technique steady state inactivation was achieved by incubation for a fixed time (12 min) with 0.25–500 μM ATP; attainment of steady state for a particular preparation of branched chain complex is shown in fig.3(a). The advantage of the steady state technique is that multiple samples may be taken at leisure for assay of active complex, total incorporation of ^{32}P and analysis of tryptic [^{32}P]phosphopeptides.

The relationship between total ^{32}P-incorporation and inactivation of the complex studied by means of the steady state technique is shown in fig.3(b). Relative to the middle portion of the curve, inactivation lags behind phosphorylation at the beginning; at the end, phosphorylation continues with little inactivation. The relationship is grossly similar to that observed in the pyruvate dehydrogenase complex in [13]. In [5], inactivation and phosphorylation appeared to be linearly correlated. Here, the basis for this apparently linear relationship in the earlier and less precise study, can be seen over the range 0–85% inactivation.

The relationship between the proportion of complex inactivated by phosphorylation and the concentration of ^{32}P in individual tryptic phosphopeptides is shown in fig.4. Fig.4(a) shows the results of experiments with the steady state technique and fig.4(b) results obtained during a time course. The results with either technique were closely similar. Relative rates of phosphorylations were TA > TB > TC and phosphorylations in TB and TC were only prominent after phosphorylation in TA reached a maximum. Phosphorylation in TA was correlated with about 66% inactivation of the complex; further inactivation was correlated with phosphorylations in TB and TC. Phosphorylations in TB and TC were apparently incomplete in these experiments and the concentration of ^{32}P attained in TB was about twice that in TA and TC. The ter-

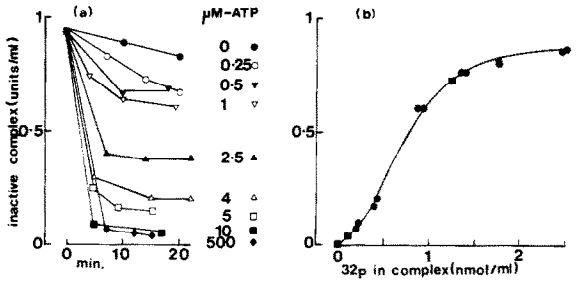

Fig.3. Technique of steady state inactivation of branched-chain complex by ATP (a); relationship between degree of inactivation and total incorporation of ^{32}P (b). In (a) complex (0.94 unit/ml) was incubated in medium B (see section 2) at 30°C with ATP at the concentrations shown, and samples assayed for complex activity. In (b) complex was incubated for 12 min at 30°C in medium B with the concentrations of ATP shown in (a). Each point is the mean of 2 obs. for complex activity and of 3 obs. for protein-bound ^{32}P. The experiment was performed twice and duplicate identical values are shown as (■).

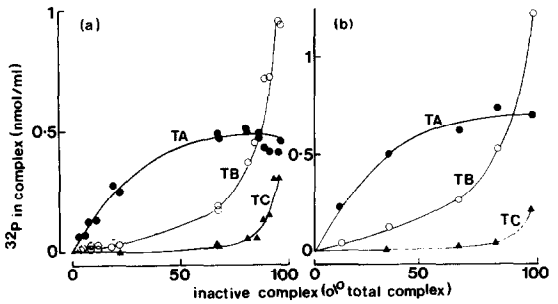

Fig.4. Relationship between inactivation of branched-chain complex by ATP and incorporation of ^{32}P from [γ-^{32}P]ATP into tryptic phosphopeptides TA, TB and TC. In (a) the experimental technique was as in fig.3; TC points between zero and 20% of inactive complex showed zero incorporation. In (b) a different preparation of branched-chain complex (4.9 units/ml) was incubated at 30°C in medium A (see section 2) with 0.5 mM [γ-^{32}P]ATP (89 dpm/pmol) and samples taken for assay of complex activity, total incorporation of ^{32}P and HVPE of tryptic phosphopeptides at 2, 5, 10, 20 and 60 min. In (a) $t_{0.5}$ for inactivation was 0.5 min and maximum incorporation was 2.8 nmol/unit; in (b) $t_{0.5}$ was 7 min and maximum incorporation was 2.35 nmol/unit.

minal pattern of ^{32}P-incorporation has been consistent in a large number of experiments (not shown). The data suggested the possibility that the increase in TC concentration was associated with a decrease in TA concentration, especially when the shoulder on peak TA was treated as a separate peak (not shown). This could not be established unequivocally in these experiments. The main problem in achieving complete incorporation is removal of contaminating ATPase activity while retaining high kinase and this problem has yet to be resolved completely.

4. CONCLUSIONS

These studies show that there is multisite phosphorylation in ox-kidney branched-chain complex and that complete inactivation may involve phosphorylation of more than one site. The results appear to show the presence of at least 3 phosphorylation sites (there may be more because, for example, TB may contain more than one site). Further interpretation requires knowledge of the stoicheiometry of phosphorylation of the α-chain of the decarboxylase component and the amino acid sequences of the tryptic phosphopeptides. Progress on these aspects has been hampered by the low concentration of the complex in tissues and unexpected difficulties in separating tryptic phosphopeptides from contaminating peptides. It has been shown here that the relationship between phosphorylations and inactivation is more complex than has been described hitherto and it has seemed important therefore to report these findings.

ACKNOWLEDGEMENTS

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

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