Dephosphorylation and reactivation of phosphorylated purified ox-kidney branched-chain dehydrogenase complex by co-purified phosphatase

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The branched-chain 2 oxoacid dehydrogenase complex has been purified from well-washed ox-kidney mitochondria together with branched-chain dehydrogenase kinase. The complex was inactivated and phosphorylated by ATP in about 5 min at 30°C. After hydrolysis of ATP by a contaminating ATPase (5–10 min) the complex was dephosphorylated and reactivated. Dephosphorylation and reactivation were linearly correlated. Reactivation was dependent upon Mg^{2+} ($K_{0.5}$ > 1 mM) and inhibited completely by 50 mM fluoride. Reactivation and dephosphorylation are attributed to a mitochondrial branched-chain dehydrogenase phosphatase.

Branched-chain 2 oxoacid dehydrogenase *Dephosphorylation* *Reactivation*
Branched-chain dehydrogenase phosphatase *BCDH complex of ox-kidney*
Mg^{2+} activation of BCDH phosphatase

1. INTRODUCTION

The branched-chain 2-oxoacid dehydrogenase complex in rat heart and skeletal muscle mitochondria in vitro and in rat heart and skeletal muscle in vivo, exists in interconvertible active and inactive forms [1–3]. Inactivation is associated with phosphorylation of the α-subunit of the decarboxylase component [4–6]. Co-purification of ox-kidney, rabbit liver and rat kidney complexes together with branched-chain dehydrogenase kinase to near homogeneity has been achieved and phosphorylation of serine residues shown [7–9]. Aspects of regulation of the kinase reaction are given in [10]. Phosphorylated complex is reactivated without dephosphorylation by a protein or protein-associated factor present in rat liver and kidney mitochondria [11]. The mitochondrial phosphatase which catalyses reactivation of phosphorylated complex by dephosphorylation has proved elusive but dephosphorylation and reactivation by a cytosolic rat-liver phosphoprotein phosphatase has been described [3]. Reactivation of phosphorylated ox-kidney branched-chain complex during dialysis was noted in [11] suggesting the possibility of co-purifying complex and phosphatase from ox-kidney mitochondria. This has been achieved in the present study which shows further that reactivation of phosphorylated ox-kidney complex by dephosphorylation requires Mg^{2+}, is inhibited by fluoride, and that dephosphorylation and reactivation are correlated, linearly.

Abbreviations: DTT, dithiothreitol; EGTA, ethanedioxybis(ethylamine) tetraacetate

2. EXPERIMENTAL

Sources of chemicals, biochemicals and [γ-^{32}P]ATP were as in [1]. Branched-chain complex was purified from ox-kidney mitochondria. The procedure was as in [7] incorporating the modification given in [10] except that NaCl was

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omitted from buffer (B). In some experiments in which activity, but not protein-bound ^{32}P, was measured, the final two steps in [7] (precipitation at pH 6.8 and at pH 6.4) which complete separation of the pyruvate dehydrogenase and branched-chain dehydrogenase complexes were omitted to increase yield. Pyruvate dehydrogenase complex has no branched-chain complex activity [11]. SDS–polyacrylamide gel electrophoresis in Tris–glycine buffer was as in [5] except that 15 cm × 14 cm gels were used.

Branched-chain complex was assayed spectrophotometrically as in [10]. One unit of complex forms 1 μmol NADH/min at 30°C. Inactivation by MgATP was at 30°C in 30 mM potassium phosphate/5 mM DTT/5 mM EGTA/10 mM $MgCl_2$/0.5 mM ATP (pH 7.5) unless stated otherwise. The concentration of branched-chain complex was between 7 and 11 units/ml. Complex incubated in the absence of ATP showed no activity change. Apparent first order rate constants for inactivation ranged from 0.7–1.5 min^{-1}. In some experiments incubations were made with [γ-^{32}P]ATP (200–874 dpm/pmol) and incorporation of ^{32}P into the complex was measured as in [12]. Adenosine triphosphatase activity (ATPase) of preparations of branched-chain complex was assayed as in [10].

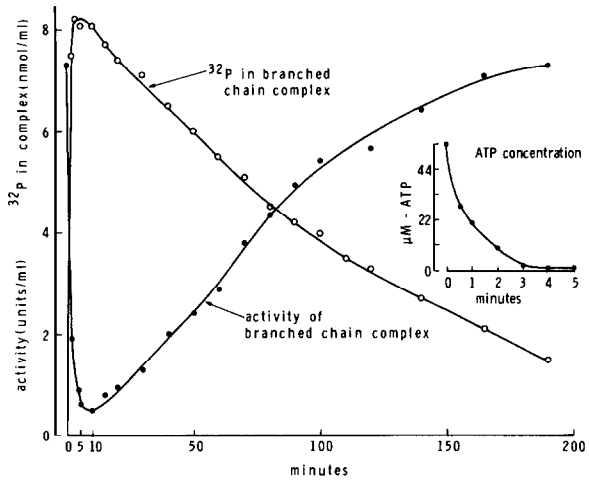

Fig.1. Phosphorylation/inactivation and dephosphorylation/reactivation of ox-kidney branched-chain complex. Highly purified ox-kidney branched-chain complex free of pyruvate dehydrogenase complex was incubated at 30°C with 50 μM [γ-^{32}P]ATP (874 dpm/pmol)/10 mM $MgCl_2$ and samples taken for assay of complex activity (2 μl) and ^{32}P incorporation (10 μl) at times shown. Concentrations of [γ-^{32}P]ATP from 0–5 min of incubation are shown in the inset figure. Each point is mean of 2 observations.

3. RESULTS AND DISCUSSION

3.1. *Reactivation and dephosphorylation of ^{32}P-phosphorylated ox-kidney branched-chain complex*

When purified ox-kidney branched-chain complex devoid of pyruvate dehydrogenase complex (7.3 units/ml) was incubated with 50 μM [γ-^{32}P]ATP (874 dpm/pmol)/10 mM $MgCl_2$, inactivation (93%) and ^{32}P incorporation (8.2 nmol/ml) were maximal in 5–10 min (fig.1). The preparation of branched-chain complex possessed ATPase activity, and hydrolysis of ATP was largely (>95%) complete in 5 min (see insert to fig.1). After 10 min of incubation reactivation and dephosphorylation of phosphorylated branched-chain complex was detected and over the course of 190 min complete reactivation of the complex was apparently achieved. Over this time dephosphorylation was incomplete (72%) (fig.1). As shown in fig.2 the disappearance of protein-bound phosphate and of inactive complex were

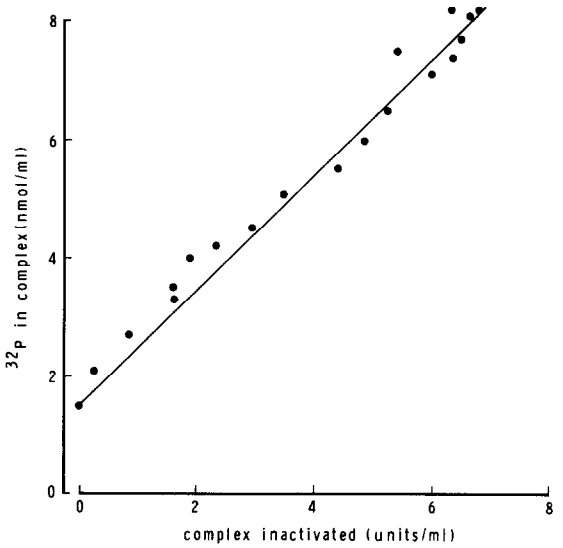

Fig.2. Correlation between concentration of inactive complex and of protein-bound ^{32}P based on data in fig.1. By least-squares linear regression analysis the slope was 1.05 ± 0.20 nmol P/unit of inactive complex and r (correlation coefficient) was 0.98 (means ± SEM).

linearly correlated (slope 1.0 ± 0.05 nmol P/unit of inactive complex, $r = 0.98$ by least-squares linear regression analysis). After apparent reactivation protein-bound phosphate was 1.54 ± 0.20 nmol P (intercept in fig.2 by least squares linear regression analysis).

Samples of the incubation were taken at 5 min (when inactivation and phosphorylation were maximal) and at 190 min (when reactivation was apparently complete) and subjected to SDS–polyacrylamide gel electrophoresis/autoradiography. The results are shown in fig.3. Autoradiographs at both time periods and at both sample loads (5 and 10 μl) showed only one band of ^{32}P. If [^{32}P]-phosphorylated pyruvate dehydrogenase complex had been present a second band of ^{32}P would be seen below the band shown in fig.3 (M_r α-chain pyruvate dehydrogenase 42200; M_r α-chain branched-chain dehydrogenase 46200 [7]). The results in fig.3 show substantial but incomplete dephosphorylation of phosphorylated branched-chain dehydrogenase between 5 and 190 min of incubation in confirmation of the results in fig.1,2.

It is suggested that apparent complete reactivation of phosphorylated branched-chain complex in association with incomplete dephosphorylation is due to the presence of phosphorylated and inactive complex in the preparation of branched-chain complex used in this study. As a consequence the concentration of phosphorylated complex during reactivation is underestimated. If this conclusion is correct then the concentration of phosphorylated complex required to be present prior to phosphorylation is about 19% of that of active complex (based on protein-bound ^{32}P at 5 and 190 min). This conclusion is supported by the results of experiments with rat liver activator protein in [11], which indicated that the preparation of branched-chain complex used in that study contained 27% of phosphorylated and inactive complex.

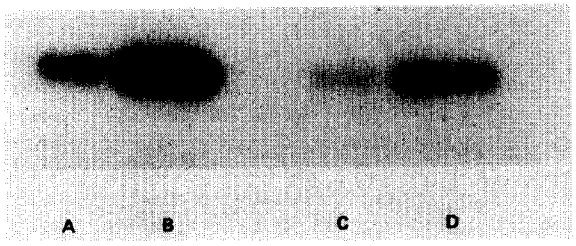

Fig.3. Autoradiograph of SDS–polyacrylamide gel electrophoresis of samples taken at 5 min (A,B) and 190 min (C,D) during the incubation shown in fig.1. Samples (10 μl) were mixed with trichloroacetic acid (to 10% w/v), the precipitates washed with 10% trichloroacetic acid to remove trichloroacetic acid-soluble ^{32}P and dissolved in SDS and 5 μl (A,C) or 10 μl (B,D) subjected to electrophoresis and autoradiography.

The results shown in fig.1 are typical of a substantial number of experiments with 7 different preparations of ox-kidney branched-chain complex, 4 of which were free of pyruvate dehydrogenase complex as a result of fractionation at pH 6.8 and pH 6.4. The phosphatase activity of the preparations was stable on storage at −80°C for at least 2 weeks (the longest period tested). The ATPase activity of preparations of branched-chain complex was lost on storage after about 4–7 days suggesting that ATPase activity is not the result of a combination of branched-chain kinase and phosphatase reactions. When ATPase activity was lost ATP hydrolysis could be induced with apyrase allowing dephosphorylation and reactivation of phosphorylated complex (not given). All of the experiments described in this paper utilised fresh preparations of complex possessing intrinsic ATPase activity and apyrase was not added. Dephosphorylation and reactivation of phosphorylated complex was either much slower or not detected when the concentration of branched-chain complex was reduced to 1 unit/ml (not given). The absence of dephosphorylation and reactivation of phosphorylated complex in [7,10,11] is explained by the much shorter period of incubation (<11 min), use of complex at 1 unit/ml, and the absence of ATPase as a result of storage prior to use.

3.2. *Effect of Mg^{2+}, Ca^{2+} and fluoride on reactivation of phosphorylated branched-chain complex*

The results of studies showing a requirement for Mg^{2+} are shown in fig.4 (left panel). Complex was phosphorylated and inactivated in 5–10 min of incubation with 0.5 mM ATP and 1 mM or 10 mM $MgCl_2$. Hydrolysis of ATP by ATPase present in the branched-chain complex was essentially complete (>97%) in 5 min at either concentration of $MgCl_2$ (shown with [γ-^{32}P]ATP; not given). Subse-

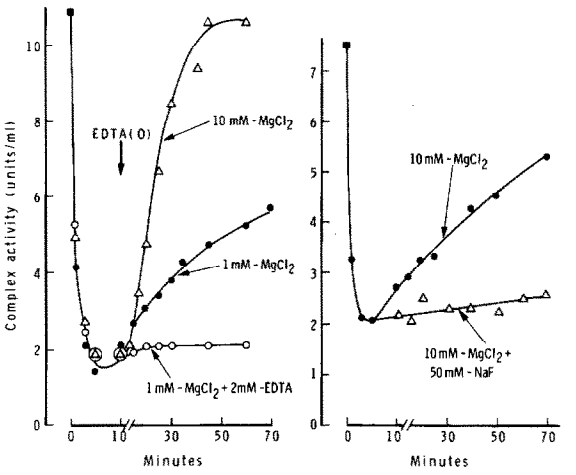

Fig.4. Reactivation of phosphorylated ox-kidney branched-chain dehydrogenase complex by dephosphorylation; effect of [$MgCl_2$] and of NaF. Purified ox-kidney complex prepared without fractional precipitation at pH 6.8 and 6.4 (see section 2) was incubated at 30°C with 0.5 mM ATP and either 1 mM or 10 mM $MgCl_2$ as shown. Samples were taken (2 μl) for assay of branched-chain complex at the times shown. In the left panel, EDTA was added to 2 mM in one incubation with 1 mM $MgCl_2$ at 10 min as shown. In the right panel, the incubate was divided after 5 min and NaF added to 50 mM to one-half. Each point is the mean of 2 observations.

quent reactivation of the complex was markedly slower with 1 mM $MgCl_2$ than with 10 mM $MgCl_2$. When 2 mM EDTA was added to incubations with 1 mM $MgCl_2$ at 10 min of incubation no obvious reactivation was detected in the course of 50 min of further incubation. It is concluded that reactivation requires Mg^{2+} and that $K_{0.5}$ is > 1 mM $MgCl_2$. The particular technique at present available does not permit accurate evaluation of $K_{0.5}$ and V_{max}. As shown in fig.4 (right panel) sodium fluoride (50 mM) inhibited completely reactivation measured in the presence of 10 mM $MgCl_2$. The results shown in fig.4 are typical of 3 different experiments with two different preparations of branched-chain complex.

The effects of $CaCl_2$ (0.6 mM and 1 mM $CaCl_2$ with 0.5 mM EGTA in place of 5 mM EGTA) on the rate of reactivation of phosphorylated branched-chain complex has been investigated employing protocols otherwise identical to those used in fig.1 and 4. No stimulation of reactivation by Ca^{2+} has been seen in any experiment (not given).

It is apparent from data given in fig.1 and 4 that the phosphatase activity varies between different preparations of branched-chain complex. In a total of 13 different studies with 10 mM $MgCl_2$, $t_{0.5}$ for reactivation ranged from 13–70 min (average 31 min).

4. CONCLUSIONS

It has been shown in this study that highly purified and phosphorylated ox-kidney branched-chain 2 oxoacid dehydrogenase complex may be fully reactivated by dephosphorylation following hydrolysis of ATP by contaminating ATPase. Reactivation (and dephosphorylation) requires Mg^{2+} and is inhibited by fluoride. It is concluded that preparations of branched-chain complex contain a branched-chain dehydrogenase phosphate phosphatase. Because the branched-chain complex was purified from extensively washed ox-kidney mitochondria it is concluded that the phosphatase is mitochondrial in origin. Reactivation in the presence of $MgCl_2$ was not stimulated further by $CaCl_2$ suggesting that the branched-chain dehydrogenase phosphatase reaction, unlike the pyruvate dehydrogenase phosphatase reaction neither requires, nor is activated by Ca^{2+}. This and other evidence reviewed in [13] may indicate non-identity of branched-chain dehydrogenase and pyruvate dehydrogenase phosphatases.

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