Regulation of the branched chain 2-oxoacid dehydrogenase kinase reaction

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1. INTRODUCTION

In a number of animal tissues there is evidence for interconvertible active and inactive forms to the mitochondrial branched-chain 2-oxoacid dehydrogenase complex (referred to here as branched-chain complex). Inactivation is correlated with phosphorylation, and activation with dephosphorylation of a protein of $M_r \sim 48\,000$ which is assumed to be the α-chain of the dehydrogenase component [1–3]. A method has been developed which yields highly purified ox kidney branched chain complex which is rapidly phosphorylated and inactivated on incubation with Mg[γ-^{32}P]ATP. This preparation is assumed to contain a branched-chain dehydrogenase kinase intrinsic to the complex [2]. This finding has been confirmed independently with purified rat kidney complex [4] and rabbit liver complex [5]. We describe here aspects of the kinetics of inactivation by MgATP and of its inhibition by branched-chain α-ketoacids, ADP and TPP; and identification of the phosphoamino acid formed.

2. EXPERIMENTAL

Isovaleryl CoA and phosphoamino acids were from Sigma (London); GTP was from BCL (Lewes). Sources of other materials were as in [2,6]. Branched-chain complex was purified as in [2] except that buffers for isolation and extraction of mitochondria were at pH 8. Preparations were totally dependent for activity on added TPP and were devoid of ATPase activity (assayed as in [7]).

Branched-chain complex was assayed spectrophotometrically as in [8] except that KCN and Triton were omitted and lipoamide dehydrogenase was increased to 30 units/ml. 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 EGTA/5 mM DTT/10 mM $MgCl_2$ (pH 7.5). Concentrations were ATP, 0.5 mM (unless given), branched-chain complex range 0.3–1 unit/ml (constant in individual experiments). Samples for assay of complex activity were taken at zero time and at 3 other times determined by the rate of inactivation (usually $t_{0.5}$, $2 \times t_{0.5}$ and $3 \times t_{0.5}$; longest period 11 min). Complex incubated in the absence of ATP showed no inactivation and phosphorylated complex incubated without ATP showed no reactivation. In some experiments incubations were made with [γ-^{32}P]ATP (255 dpm/pmol) and incorporation of ^{32}P into complex (at 1, 3 and 5 min) measured as in [9].

SDS–PAGE of complex and of [^{32}P]phosphorylated complex and autoradiography was as in [8]. For identifaction of [^{32}P]phosphoamino acids branched-chain complex (1.7 units in 0.6 ml) was incubated for 15 min as above with 0.5 mM [γ-^{32}P]ATP (1054 dpm/pmol) (94% inactivation). Tryptic phosphopeptides were prepared as in [9], chromatographed in 25 mM HCl on Sephadex G-10, and heated at 110°C in 6 N HCl/2 mg/ml^{-1} phenol. Samples taken at 3, 15, 45 and 90 min were freeze-dried, taken up in aqueous 0.5% (v/v) pyridine/5% (v/v) acetic acid (pH 3.5) and electrophoresed in the same buffer on Whatman 3MM paper

Abbreviations: DTT, dithiothreitol; ketoleucine, 4-methyl-2-oxopentanoate; ketovaline, 3-methyl-2-oxobutyrate; ketoisoleucine, 3-methyl-2-oxopentanoate; TPP, thiamin pyrophosphate; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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(30 min, 3.5 kV; ~18 000 dpm in ^{32}P/sample). Markers of $^{32}P_i$ (15 000 dpm) and of phosphoamino acids (50 nmol) were included in each electrophoresis. Phosphoamino acid markers were detected with ninhydrin and ^{32}P by autoradiography. Solutions of branched-chain ketoacids, CoA, acetyl CoA, isovaleryl CoA, NAD^+, NADH, ATP and [γ-^{32}P]ATP were standardized as in [6, 8–10]; ATP in ADP and GTP was assayed with luciferase [11].

Inactivation of branched-chain complex by MgATP is pseudo first-order [2] and relative rates of inactivation were calculated as the apparent first-order rate constant by least-squares linear regression analysis. In all such analyses the correlation coefficient (r) was >0.97 and deviations from linearity were not significant ($P>0.05$). In computing values for K_m [12] or K_i [13,14] apparent first-order rate constants were used in place of initial velocities (in any one experiment inital concentrations of complex were constant; and ATP concentration during incubation changed by $<10\%$). MgATP concentrations were assumed to be equivalent to ATP concentration (at 0.5 mM ATP, 98.5% of ATP was computed to be MgATP and at 14 μM ATP, 91.5% was computed to be MgATP). In the holo-complex reaction K_m-values for TPP and ketoacids were computed as in [12] from initial velocities.

3. RESULTS AND DISCUSSION

3.1. *Phosphorylation and inactivation of purified branched-chain complex*

Results typical of SDS-PAGE of these [^{32}P]phosphorylated complexes (Coomassie blue and autoradiogram) are shown in fig.1. Three major protein bands were detected corresponding to the α- and β-chains of the branched ketoacid dehydrogenase component and the acyltransferase [15]. A single band of ^{32}P contained with the α-chain of the dehydrogenase component. Some preparations have shown (Coomassie blue) minor contamination with other protein bands of higher M_r (one such band is in fig.1) but only one band of ^{32}P. At a saturating concentration of MgATP (0.5 mM, 40 × K_m– see table 1) apparent first-order rate constants (min^{-1}) for inactivation of 7 preparations of complex ranged from 0.08 ± 0.001–1.12 ± 0.04 min^{-1} (mean ± SEM for 4 time points); the mean value was 0.50. It is suggested that differences in rates of inactivation by ATP may reflect variable loss of kinase during purification of different preparations

Table 1

Inactivation of ox kidney branched-chain 2 oxoacid dehydrogenase complex by ATP; inhibition by ketoacids, ADP and TPP

Inhibitor	[MgATP] (μM)	n	[Inhibitor]	K_m or K_i (mean ± SEM)	Type
–	14,20, 33,50,300,520	14	–	12.6 ± 1.04 μM K_m	
Ketoleucine	As above	19	0.5 mM	0.48 ± 0.06 mM K_i	NC
Ketovaline	As above	6	4.1 mM	8.9 ±3.2 mM K_i	NC
D,L-Keto isoleucine	14,20,33,50,300	5	0.75 mM	0.92 ± 0.14 mM K_i	NC
ADP	28,42,48,61,78	12	1.0 mM	0.27 ± 0.03 mM K_i	C
Thiamin pyrophosphate	14,20,33,50	7	20 μM	5.9 ±2.34 μM K_i	NC
				4.0 ±1.0 μM K_i	UC

Purified ox kidney branched-chain complex was incubated at 30°C with MgATP at concentrations shown ± the inhibitors at the concentrations shown. Complex activity was assayed at zero time and at 3 other times, apparent first-order rate constant for inactivation determined, and K_m and K_i values computed as in section 2. The total number of incubations (n) at all MgATP concentrations is shown in column 3. *Type:* NC = non competitive inhibition; C = competitive inhibition; UC = uncompetitive inhibition. With ADP, MgATP concentrations incorporate ATP present in ADP (assayed with luciferase)

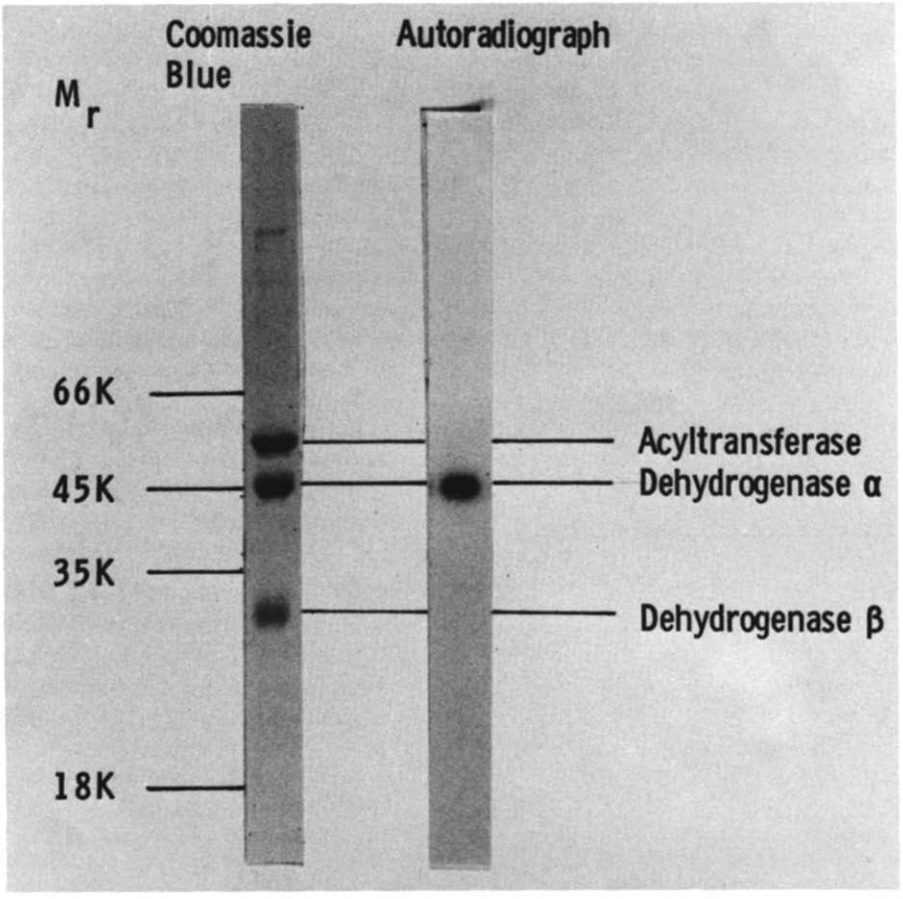

Fig.1. SDS–polyacrylamide gel electrophoresis of [^{32}P]phosphorylated ox kidney branched-chain 2 oxoacid dehydrogenase complex. Branched-chain complex (1 unit/ml) was incubated at 30°C for 60 min with 0.5 mM ATP (390 dpm/pmol), precipitated and washed with trichloroacetic acid, taken up in SDS, neutralized, heated at 100°C with mercaptoethanol and electrophoresed in 12% polyacrylamide slabs in Tris/glycine buffer as in [7]. Track A is Coomasie blue stain (M_r-values, mean ± SEM for 3 obs. 52 000 ± 601, 46 000 ± 452 and 31 000 ± 449); track B is autoradiograph.

of the complex. If so, the loss of kinase occurs during precipitation of contaminating material at pH 6.8, the penultimate step in purification in [2]. The rate constant for inactivation prior to this step was consistently ~ 1 (not shown). As in [2] inactivation was correlated closely with incorporation of ^{32}P from [γ-^{32}P]ATP (not shown).

The only phosphoamino acid detected after acid hydrolysis of tryptic [^{32}P] phosphopeptides from [^{32}P]phosphorylated complex was phosphoserine (fig.2). The results in fig.2 are typical of experiments with two different [^{32}P]phosphorylated complexes. It is concluded that inactivation by MgATP is correlated with phosphorylation of a serine residue or residues.

The K_m for inactivation by MgATP was 12.6 ± 1.04 μM (mean ± SEM) (table 1). The complex was also inactivated by 1 mM ADP (rate constants, min^{-1}, 1 mM ADP 0.11; 0.5 mM ATP, 0.33) and by 1 mM GTP (rate constants, min^{-1}, 1 mM GTP GTP 0.18; 0.5 mM ATP, 0.45). The effects of ADP and GTP could be due to contamination with ATP. By luciferase assay ADP contained 2.8% of ATP and GTP contained 0.9% of ATP (mol/mol). Based on the K_m for ATP and the K_i for ADP given in table 1, the rate constants expected for inactivation

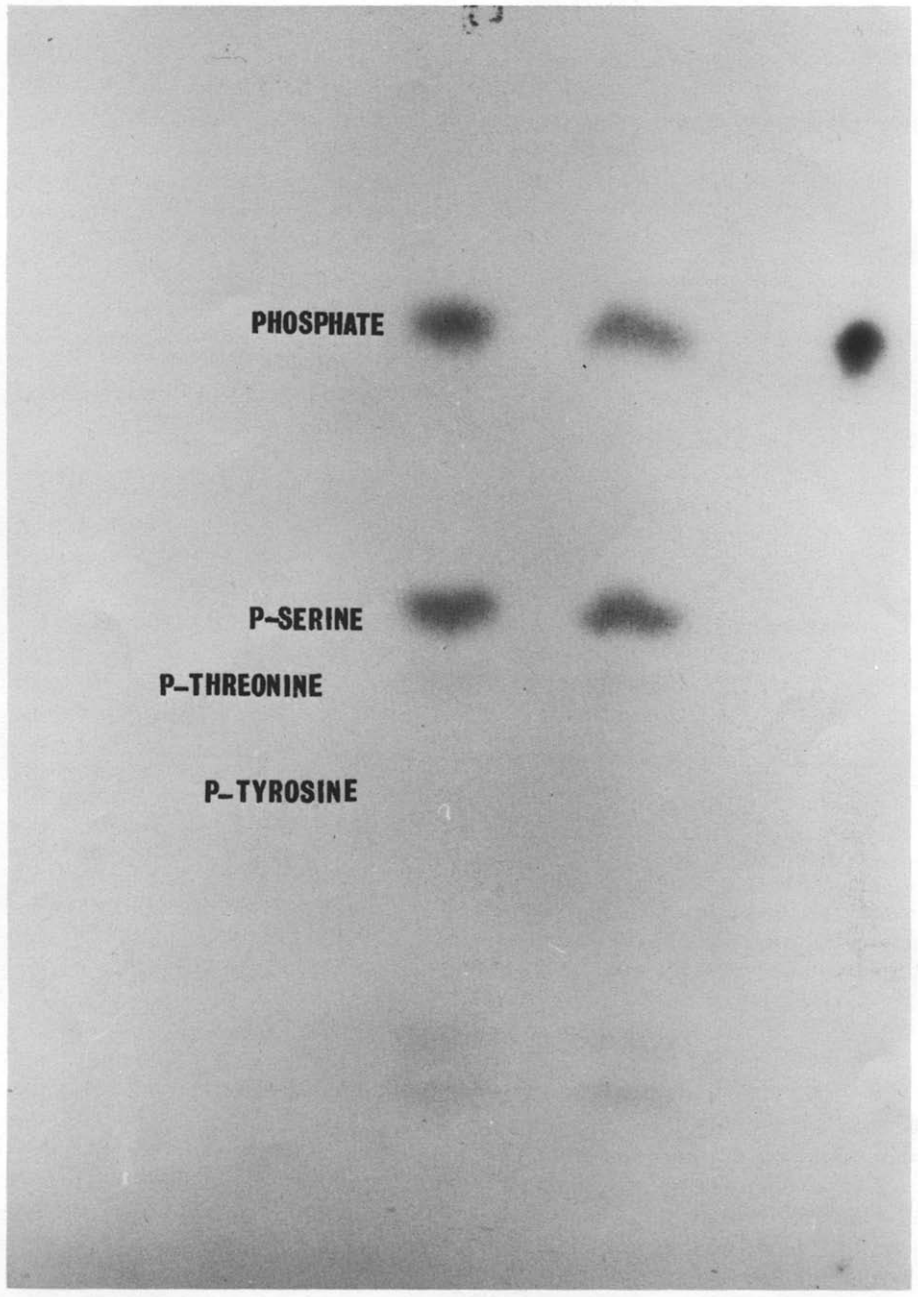

Fig.2. Autoradiograph of high-voltage paper electrophoresis (pH 3.5) of an acid hydrolysate of a tryptic digest of [^{32}P]phosphorylated branched-chain 2-oxoacid dehydrogenase complex. Tracks 1 and 2 are 90 min acid hydrolysate. Track 3 is $^{32}P_i$ marker. The positions of marker phosphoamino acids added to one hydrolysate and run also in separate tracks were as shown.

by ATP in ADP or GTP were 0.11 and 0.19 min^{-1}, respectively. These computed rate constants show excellent agreement with those observed experimentally. Trace contamination of GTP by ATP is difficult to show unequivocally.

3.2. *Effects of branched-chain ketoacids, ADP, thiamin pyrophosphate, CoA, acetyl CoA, isovaleryl CoA, NAD^+ and NADH on the branched-chain 2-oxoacid dehydrogenase kinase reaction*

As shown in table 1 inactivation of branched-chain complex was inhibited non-competitively by branched chain α-ketoacids. The values for K_I were much greater than the corresponding K_m values for the holo-complex reaction determined on the same preparation (ketolecuine 8.7 ± 0.17 μM; D,L-ketoisoleucine, 9.8 ± 1.36 μM; ketovaline, 16.2 ± 1.52; mean ± SEM for 16–29 obs.). These values for K_m are lower than those for ox kidney complex [15] but comparable to those obtained in this laboratory for ox liver complex [10]. Possible reasons for this difference are that solutions of branched-chain α-ketoacids were standardized enzymically here or differences in the programmes used to calculate K_m. Inactivation of the complex by MgATP was inhibited competitively by ADP, and non-competitively (or uncompetitively) by TPP (table 1). The K_i for TPP in the kinase reaction was comparable to the K_m for TPP in the holo-complex reaction (3.2 ± 0.24 μM; mean ± SEM for 11 obs.). Here, we have preferred to monitor the branched-chain kinase reaction by following inactivation by MgATP as opposed to incorporation of [^{32}P][γ-^{32}P]ATP into the complex. This is because the number of phosphorylation sites and their function is unknown; the results of preliminary studies suggest that >1 tryptic [^{32}P]phosphopeptide may be formed fully phosphorylated complex (not shown). Inhibition of ^{32}P incorporation into the complex from [γ-^{32}P]ATP by branched-chain α-ketoacids and by TPP has been demonstrated (not shown).

The rate of inactivation of branched chain complex by 0.5 mM ATP was not changed by CoA or acetyl CoA (not shown). In these experiments 7 mixtures of CoA acetyl CoA were used; the total concentration (CoA + acetyl CoA) was 1.15 mM and concentration ratios ranged from 1.15 mM CoA/zero acetyl CoA to zero CoA/1.15 mM acetyl CoA. In comparable experiments with CoA and isovaleryl CoA the only effect observed was some acceleration of inactivation with 0.23 mM CoA/0.92 mM isovaleryl CoA and with zero CoA/1.15 mM isovaleryl CoA. The rate constants were increased from 0.51 ± 0.02 min^{-1} (ATP alone) to 0.61 ± 0.02 and 0.67 ± 0.02 min^{-1}, respectively (mean ± SEM for 3 incubations, 4 time points each incubation). This effect of isovaleryl CoA was not seen in the presence of 6 mM NAD^+/1 mM NADH/lipoamide dehydrogenase (20 units/ml) (not shown). The effects of NAD^+ (7 mM), NADH (7 mM) and of mixtures of (NAD^+ + NADH; total conc. 7 mM) on the branched-chain ketoacid dehydrogenase kinase reaction was investigated with 0.5 mM ATP and lipoamide dehydrogenase (20 units/ml). In a single experiment (of 4 performed) the rate constant for inactivation was approximately halved by 7 mM NAD^+; the effect of NAD^+ was not reversed by NADH (3.5 mM NAD + 3.5 mM NADH). In the other 3 experiments NAD^+ had no effect. The rate of inactivation of branched-chain complex by 0.5 mM ATP was not influenced by NADH (not shown).

4. CONCLUSIONS

The method for purifying ox kidney branched-chain complex with intrinsic kinase activity in [2] is reproducible. Loss of kinase activity was associated with purification steps at < pH 7; unfortunately such steps have been necessary to separate the branched-chain complex from the pyruvate dehydrogenase complex. The preparations used in the present study were highly purified as judged by SDS–PAGE of complex and of [^{32}P]phosphorylated complex.

The branched-chain dehydrogenase kinase reaction was inhibited by branched-chain 2-oxoacids, ADP and TPP, and is analogous to the pyruvate dehydrogenase kinase reaction which is inhibited by pyruvate, ADP and TPP. The K_i-values for branched-chain 2-oxoacids for the branched-chain kinase reaction were high and comparable to the K_i for pyruvate in the pyruvate dehydrogenase kinase reaction. The K_i value for ADP (270 μM) was higher than for the pyruvate dehydrogenase complex (80 μM in [7]). The pyruvate dehydrogenase kinase reaction is inhibited by CoA and NAD^+ and activated by acetyl CoA and NADH [6, 17]; these effects were initially elusive and difficult to demonstrate.

No comparable effects of CoA, NAD^+, acetyl CoA, isovaleryl CoA or NADH have been seen here employing conditions which are successful with the pyruvate dehydrogenase kinase reaction. A major problem with the branched-chain complex is the relative lack of information about physiological factors which may modulate interconversions in the branched-chain complex in vivo or in isolated mitochondria.

The K_m for ATP (12.6 μM) in the branched-chain ketoacid dehydrogenase reaction is comparable to that for pyruvate dehydrogenase kinase and many other protein kinases, and phosphoserine was the only phosphoamino acid detected as in the vast majority of protein phosphorylations. Inactivations by GTP was observed (see also [5]) and GTP reported to be a substrate for 2 glycogen synthase kinases [16]. Because of the low K_m for ATP it is possible that these effects of GTP were due to contamination with ATP.

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