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EFFECTS OF CONDITIONS FAVORING ENZYME PHOSPHORYLATION AND DEPHOSPHORYLATION ON THE ACTIVITY OF THE α-KETO ACID DEHYDROGENASES, WITH PARTICULAR REFERENCE TO THE BRANCHED-CHAIN α-KETO ACID DEHYDROGENASE ACTIVITIES(†)

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Summary:

These studies have shown that in the crude system of rat liver mitochondria the branched-chain α-keto acid dehydrogenase activities are activated at high (10.0mM) Mg^{++} concentrations favoring dephosphorylation, and are inactive at low (1.0mM) Mg^{++} concentrations favoring phosphorylation. In this crude system, α-Ketoglutarate dehydrogenase activity was also regulated in this manner. In general, the optimum Mg^{++} and ATP levels for activation were 10mM and 1.0mM respectively.

INTRODUCTION:

Because of the integral role that the pyruvate and α-ketoglutarate dehydrogenase complexes play in the oxidation of pyruvate and other substrates through the tricarboxylic acid cycle, they have both been isolated, purified and well-characterized from mammalian tissues (1-5). When pyruvate dehydrogenase complex (PDC)* is phosphorylated by a specific

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* The following abbreviations have been used: PDC, pyruvate dehydrogenase complex; α-KgDC, α-ketoglutarate dehydrogenase complex; Py, pyruvate; α-Kg, α-ketoglutarate; KIV, α-ketoisovalerate; KIVDC, α-ketoisovalerate dehydrogenase complex; KIC, α-ketoisocaproate; KICDC, α-ketoisovalerate dehydrogenase complex; KMV, α-keto-β-methylvalerate; KMVDC, α-keto-β-methylvalerate dehydrogenase complex.

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protein kinase under suitable conditions it is inactive, but can be re-activated by dephosphorylation by a specific phosphatase (6,7). This has been shown to be true in tissues and mitochondria (8) as well as with the purified enzyme complex (9,10,11). Phosphorylation (and inactivation) are favored at low Mg^{++} concentrations accompanied by high energy charge (or high ATP/ADP ratio), high NADH/NAD^+ ratio and high free fatty acid levels, (12,13,14,15) while dephosphorylation (activation) is favored at high (10 mM) Mg^{++} concentration and low energy charge, low free fatty acid levels or low NADH/NAD^+ ratio. This appears to constitute an important mechanism for regulation of the amount of pyruvate entering the tricarboxylic acid cycle. It has been claimed that purified α-ketoglutarate dehydrogenase complex (αKgDC)* is not subject to regulation by phosphorylation-dephosphorylation (16).

The corresponding enzymes (5) responsible for the oxidative decarboxylation of the branched-chain α-ketoacids, α-ketoisovalerate, (KIV)* α-ketoisocaproate (KIC)* and α-keto-β-methylvalerate (KMV)*, have not as yet been significantly purified and characterized. They are presumably also located on the inner membrane of the mitochondria (17) and occur as a multienzyme complex analogous to PDC and α-KgDC. As yet no study has been reported to indicate whether these enzyme activities are also subject to activation-inactivation under conditions which favor dephosphorylation-phosphorylation for PDC in mitochondria. It is the purpose of this study to investigate in rat liver mitochondria, the effects of Mg^{++} and ATP concentrations, other known cofactors, and assay conditions on the total α-ketoacid dehydrogenase activity and the proportion in the active state under the conditions studied for the three branched-chain α-ketoacids as substrates and for αKg and Py for comparison.

MATERIALS AND METHODS

Dithiocrythritol, cold pyruvate, α-ketoglutarate and branched-chain α-ketoacids as sodium salts, Dowex 50 WX4-400 resin, catalase, L-amino acid oxidase, ATP, and Hyamine hydroxide were obtained from Sigma

Chemical Co., St. Louis, MO. The 1-^{14}C-labeled branched-chain α-amino acids (L-leucine, L-valine and L-Isoleucine) and 1-^{14}C-pyruvate and 1-^{14}C-α-ketoglutarate were obtained from Biochemical and Nuclear Corp., Burbank, CA. All other chemicals were highest purity available.

1-^{14}C-labeled branched-chain α-ketoacids were prepared from the corresponding α-amino acids essentially by the method of Ruediger et al. (18). The eluate from the Dowex 50 WX4 resin column (H^+-form) was neutralized and diluted to give approximately 7 x 10^6 counts per minute/ml and stored at -20° C until used.

Mitochondria were prepared from freshly obtained livers of normal male Sprague-Dawley rats essentially by the procedure of Schneider and Hogeboom (19). The resulting mitochondrial pellet was then resuspended in a volume of 0.25 M sucrose to make the total volume in ml equal to the weight of the liver used in grams.

The assay for α-ketoacid dehydrogenase activity was accomplished by an adaptation of a method suggested by J. R. Moskal and S. Basu for measurement of glutamate decarboxylase activity (20). A volume of mitochondrial suspension containing 0.4 to 0.6 mg protein was placed in a mixture of 18 μmoles $KH_2PO_4^-$-$KHPO_4^=$ buffer, pH 7.0; 9 μmoles dithioerythritol; 0.45 μmole $^+$ATP, except where indicated; 0.9 or 9 μmoles $MgCl_2$ in a total volume of 0.9 ml. After incubation for 10 min, at 37°, 0.09 ml aliquots of this solution were placed in small 6 x 5 mm test tubes and 10 μl of the appropriate ^{14}C-α-ketoacid added to give a final concentration of 5 mM and 35-40,000 cpm. This small 6 x 50 mm tube was then carefully placed in a 17 x 100 mm polystyrene tube in which a 3.8 x 7.5 cm strip of Whatman 3 MM chromatographic grade filter paper had been placed, which had been spotted on the top end with 25 μl of a 1.0 M solution of Hyamine hydroxide. The tube was capped tightly with a rubber serum stopper and incubated in a water bath with shaking for 60 minutes at 37° after which the reaction was stopped by carefully injecting 50 μl of a solution of 0.2% 2,4-dinitrophenylhydrazine in 5 N H_2SO_4 through the stopper into the small inner tube. The tube was then shaken for 30 minutes to facilitate transfer of liberated $^{14}CO_2$ into the Hyamine on the paper strip. The top of the strip with the Hyamine spot was cut off and placed in a scintillation vial containing 10 ml of a scintillation fluid composed of 4 g Omnifluor (New England Nuclear) and 50 ml Triton X-100 in 1000 ml Toluene. The vials were then allowed to stand at least 2 hours in the dark to reduce chemiluminescence, after which the radio-activity due to $^{14}CO_2$ was counted in an appropriate liquid scintillation counter. Blanks without mitochondria and samples were all run in triplicate. Proteins in the mitochondrial suspensions were determined by the method of Lowry et al. (21). From the protein, the sample cpm-blank cpm, and the specific activity of the 1-^{14}C-α-ketoacid substrates used, the activity of the α-ketoacid dehydrogenase was calculated in μmoles CO_2 liberated/min./mg protein. Mg^{++} and ATP concentrations were varied as shown in individual experiments.

RESULTS AND DISCUSSION

As shown in Table I, with intact rat liver mitochondria in the presence of either 0.1 mM or 0.5 mM ATP at a low Mg^{++} level (1.0 mM), only a small fraction of the total enzyme activity is obtained with all five of the substrates studied. When the Mg^{++} concentration is increased to 10.0 mM at the same ATP concentration, a 3-to-5-fold increase in

TABLE I - EFFECTS OF LOW AND HIGH Mg^{++} CONCENTRATIONS ON THE ACTIVATION OF THE α-KETOACID DEHYDROGENASES AT TWO LEVELS OF ATP

α Ketoacid Substrate	Activity (nMoles CO_2/min./Mg Protein)*			
	at 0.1 mM ATP		at 0.5 mM ATP	
	1.0 mM Mg^{++}	10 mM Mg^{++}	1.0 mM Mg^{++}	10 MM Mg^{+}
Pyruvate	0.27 ± 0.022	2.98 ± 0.558	0.48 ± 0.051	6.01 ± 0.494
α-Ketoglutarate	1.75 ± 0.243	4.75 ± 0.558	3.67 ± 0.479	10.33 ± 1.022
α-Ketoisovalerate	0.83 ± 0.080	2.10 ± 0.273	1.18 ± 0.068	4.87 ± 0.530
α-Ketoisocaproate	0.36 ± 0.034	0.72 ± 0.069	0.49 ± 0.059	1.92 ± 0.226
α-keto-β Methylvalerate	0.48 ± 0.043	1.06 ± 0.262	0.59 ± 0.060	3.49 ± 0.397

* Mean ± std. error for 13 rats. Assay conditions: Phosphate buffer 0.02 M, pH 7.0(18 μ moles); dithioerythritol, 9 μ moles; ATP, 0.09 and 0.45 μ moles; and 0.9 or 9 μ moles of $MgCl_2$ in a total volume of 0.9 ml. After incubation for 10 min. at 37° 0.09 ml aliquots were taken for assay, to which 0.01 ml of the 1-^{14}C-α-Keto Acid was added containing 0.5 μ mole and 40,000 Cpm.

activity is obtained with all five substrates. The results with pyruvate confirm those reported by many others for purified PDC (6,7) and for PDC in liver mitochondria (8). Although it has been claimed (16) that purified α-KgDC does not show this phenomenon of activation with increasing Mg^{++} concentration, it does show this in the crude system used here. With purified αKgDC, the kinase and phosphatase may have been removed in the purification steps, and hence the purified αKgDC might be initially in the active form. The activities of the branched-chain α-ketoacid dehydrogenase complex(es) (KIVDC, KICDC and KMVDC) also all appear to be regulated by this mechanism.

Since the Mg^{++} concentration so markedly affects the activity, it seemed important to test the effects of a wider range of Mg^{++} concentrations on the activities. The results are shown in Figure 1. In general, the 1 mM added Mg^{++} concentration gives about the same activity as with no added Mg^{++}.

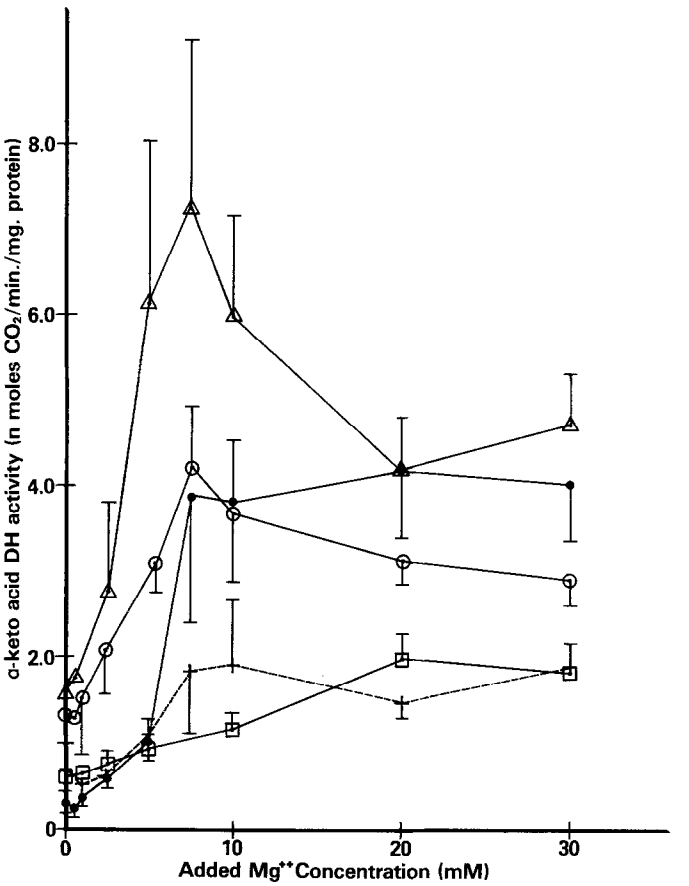

Figure 1. Effect of varying added Mg^{++} concentrations at constant ATP (0.5 mM) concentration on the α-ketoacid dehydrogenase activities with pyruvate (Py=•); α-ketoglutarate, (αKg=△); α-ketoisovalerate (KIV=⊙); α-ketoisocaproate; (KIC=⊡); and α-keto-β-methylvalerate (KMV=+) as substrates. Assay conditions as in Table I, except that Mg^{++} was varied as indicated. Bars show the standard errors.

The optimum level of Mg^{++} for activation is generally between 7.5 and 10 mM added Mg^{++}. With αKg, Mg^{++} concentrations above 10 mM are inhibitory. With KIC, the maximum activity is not attained until 20 mM Mg^{++} is added. With KIV, KMV and Py, the activity remains essentially constant above 10 mM Mg^{++}. These data indicate that the Mg^{++} concentrations that have generally been used to favor inactivation (phosphorylation) i.e. 1.0 mM and activation (dephosphorylation), i.e. 10 mM, are optimum and apply also with the branched-chain α-ketoacids as the substrates.

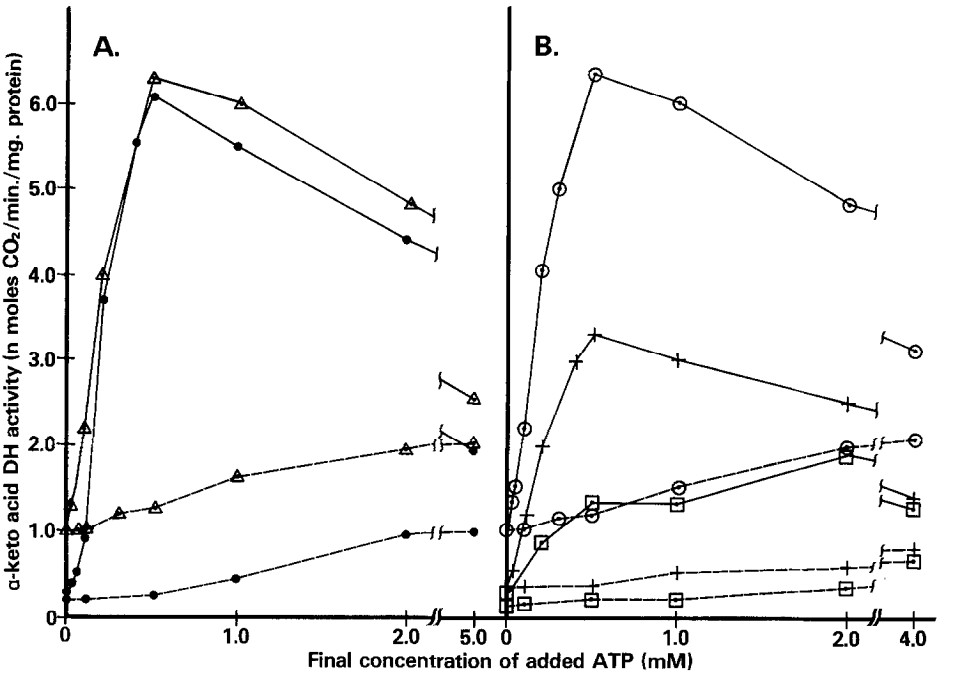

Figure 2. Effect of varying added ATP concentrations on the α-ketoacid dehydrogenase activities at 1.0 (---) and 10.0 (——) mM concentrations of Mg^{++}. Assay conditions as in Table I except ATP varied as indicated.

(A) With Py (•) and αKg(△) as substrates.

(B) With KIV (⊙), KIC (⊡) and KMV (+) as substrates.

In this respect it also seemed important to check the effects of varying ATP concentrations on the activities with the various substrates. The results are presented in Figures 2A and 2B. With Py, KIC and KIV as substrates, the optimum ATP level for activation by 10 mM Mg^{++} is 0.5 mM. For αKg the optimum appears to be 1.0 mM and for KMV, 2.0 mM. The total Mg^{++}, bound ATP-Mg^{-} and total ATP were not determined in these preparations of mitochondria, so the concentrations indicated refer only to the amounts of ATP and Mg^{++} added.

It would thus appear that this mechanism is a general one for regulation of the activities of the α-Keto Acid dehydrogenases in liver.

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