Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 74-81

ACTIVITY STATE OF THE BRANCHED CHAIN α-KETOACID DEHYDROGENASE COMPLEX IN HEART, LIVER, AND KIDNEY OF NORMAL, FASTED, DIABETIC, AND PROTEIN-STARVED RATS

Sarah E. Gillim, Ralph Paxton, George A. Cook, and Robert A. Harris

Department of Biochemistry, Indiana University School of Medicine, Indianapolis, IN 46223

Received January 6, 1983

SUMMARY. The proportion of active (unphosphorylated) branched chain α-ketoacid dehydrogenase was determined in tissues from rats in different metabolic states. Hearts from normal, high-protein, and low-protein fed rats contained about 45% of the enzyme in the active form. Only 10-20% of the enzyme was active in hearts of fasted and diabetic rats. Virtually all of the liver enzyme was in the active form in fed, fasted, diabetic and high-protein fed animals. Protein starved rats, however, exhibited a dramatic decrease in both the % active form and total amount of liver enzyme. Kidneys from normal, fasted, diabetic and high-protein fed rats contained 70-80% of the enzyme in the active form. The % active form of the kidney enzyme decreased in protein starved rats, but less dramatically than in liver. Covalent modification is concluded to be important for *in vivo* regulation of the branched chain α-ketoacid dehydrogenase complex.

INTRODUCTION. The branched chain α-ketoacid dehydrogenase complex is subject to *in vitro* covalent modification, with inactivation by phosphorylation and activation by dephosphorylation (1-6). The kinase responsible for inactivation purifies with the complex (1,2,4) but the phosphatase responsible for activation is lost during extraction (2,4). Studies from this laboratory (5,6) have shown that a broad specificity protein phosphatase is capable of dephosphorylating the branched chain α-ketoacid dehydrogenase complex to produce full enzyme activity. This phosphatase makes it possible to measure the proportion of the complex in the active form under various metabolic conditions. The total activity present in a tissue can now be measured and the significance of covalent modification in regulating the complex under various hormonal and nutritional states can be appraised.

MATERIALS AND METHODS. Materials. Biochemicals were from Sigma Chemical Company. The 8% and 64% protein diets were from ICN Nutritional Biochemicals, catalog numbers 904667 and 904669, respectively.
Rat Care. Male Wistar rats, 200-300 g, were used. Diabetes was induced by intravenous injection of either 65- or 150 mg streptozotocin/kg body weight. The larger dose was used to produce highly ketotic insulin-dependent animals (7). The rats were kept 48 h on normal laboratory chow before use. All dia-

0006-291X/83/040074-08$01.50/0

betic rats had blood glucose values greater than 400 mg/dl, as measured with a Beckman Glucose Analyzer 2. Liver acetoacetate and β-hydroxybutyrate were measured by the method of Williamson et al. (8).

Fasted rats were without food for 48 h. Rats received the low- and high-protein diets for 12 days. The normal laboratory chow diet (Wayne Lab-Blox) was 27% in protein.

Tissue Preparation and Enzyme Assay. Rats were killed by cervical dislocation. Heart, liver, and kidneys were removed in that sequence (approximately 30 seconds) and freeze-clamped at liquid nitrogen temperature. Extracts were prepared essentially by the procedure of Parker and Randle (9). Liver and heart branched chain α-ketoacid dehydrogenase was extracted by freeze-thawing the powdered tissue three times in 5 volumes of ice-cold 30 mM KPi, pH 7.5, 3 mM EDTA, 5 mM dithiothreitol, 0.5 mM α-ketoisocaproate, 1 mM N-α-tosyl-l-lysine chloromethyl ketone (protease inhibitor), and 5% (v/v) Triton X-100, followed by homogenization with a Polytron PT-10 homogenizer for 20 seconds at low speed (setting of 3). Kidneys were extracted in the same manner except that 3% bovine serum was used as protease inhibitor. The homogenates were centrifuged at 33,000 x g for 5 min at 4° C. The supernatants were collected and centrifuged at 180,000 x g for 90 min. The pellets were dissolved by gentle homogenization in 1 ml of 50 mM HEPES, pH 7.0, 0.5 mM dithiothreitol, and 0.1% (v/v) Triton X-100. To activate the complex by dephosphorylation, 20 µl of a broad specificity protein phosphatase was incubated for 15 min at 37° C with 50 µl of extract. This phosphatase preparation (24 mg protein/ml and 15.6 milliunits alkaline phosphatase activity/mg protein) was similar to that used previously (5) but was not taken through the DEAE-A50 chromatography step. Branched chain α-ketoacid dehydrogenase activity was assayed spectrophotometrically as described previously (5).

RESULTS. Activity State of Branched Chain α-Ketoacid Dehydrogenase in Heart. The % active form of branched chain α-ketoacid dehydrogenase in heart extracts was determined by dividing the activity of the enzyme prior to incubation with the broad-specificity phosphatase by the activity following incubation with the phosphatase (Table 1). This procedure has been used previously in this laboratory (5,6) to show a direct correlation between the capacity of the perfused rat heart to oxidize leucine and the activity state of the branched chain α-ketoacid dehydrogenase complex. Hearts from 48 h fasted and streptozotocin-treated rats contained substantially less of the enzyme in the active form than fed animals (Table 1). The animals made diabetic with the low-dose of streptozotocin had the lowest % active form. Those made diabetic with a high-dose appeared to have decreased total enzyme activity. The severity of the diabetes was greater, as expected, with the high-dose of streptozotocin (liver ketone bodies 0.58 ± 0.03, 4.8 ± 0.8, and 9.1 ± 0.9 µmol/g wet wt, mean ± S.E.M. for control, low-dose and high-dose animals, respectively).

Activity State of Branched Chain α-Ketoacid Dehydrogenase in Liver. Only slight activation with phosphatase was observed with branched chain α-ketoacid

TABLE 1. Proportion of the Branched Chain α-Ketoacid Dehydrogenase Complex in the Active Form in Various Tissues of Rats in Different Metabolic States

Tissue	Metabolic State (n)	Enzyme Activity[a] -Pase	Enzyme Activity[a] +Pase	% Active Form
		milliunits/g wet wt		
Heart	Fed (10)	128 ± 10	274 ± 24	48 ± 3
	Fasted (5)	52 ± 7*	332 ± 33	16 ± 2*
	Low Protein (10)	111 ± 16	244 ± 19	44 ± 5
	High Protein (4)	124 ± 8	286 ± 36	44 ± 3
	Low Streptozotocin (6)	27 ± 3*	260 ± 16	10 ± 1*
	High Streptozotocin (8)	52 ± 10*	217 ± 13*	24 ± 4*
Liver	Fed (10)	612 ± 25	649 ± 33	94 ± 3
	Fasted (5)	661 ± 48	654 ± 62	102 ± 3
	Low Protein (10)	65 ± 15*	189 ± 18*	33 ± 5*
	High Protein (4)	643 ± 48	662 ± 83	99 ± 5
	Low Streptozoeocin (6)	653 ± 45	635 ± 44	103 ± 3
	High Streptozotocin (8)	438 ± 30*	431 ± 33*	102 ± 2
Kidney	Fed (9)	258 ± 18	341 ± 22	77 ± 3
	Fasted (4)	208 ± 12	293 ± 18	71 ± 3
	Low Protein (10)	200 ± 18*	385 ± 35	54 ± 4*
	High Protein (4)	332 ± 30*	471 ± 47*	70 ± 5
	Low Streptozotocin (6)	305 ± 20	390 ± 18	78 ± 5
	High Streptozotocin (6)	223 ± 15	306 ± 20	73 ± 2

[a] One milliunit of activity refers to 1 nmol of NADH produced per min. All values are means ± S.E.M. for extracts prepared from the number of rats indicated. ±Pase refers to whether extract incubated with phosphatase prior to assay of activity.

* Statistically significant ($P < 0.05$) from control (fed) group by analysis of variance and two-tailed Student's t-test.

dehydrogenase extracted from livers of fed animals (Table 1). This raised the question of whether the broad specificity phosphatase was effective with the liver enzyme. It was found, however, that the liver enzyme can be reactivated by the phosphatase following inactivation with ATP (Fig. 1). These results show that any inactive (phosphorylated) enzyme present in extracts prepared from liver by the procedure used in this study could be readily activated (dephosphorylated) by treatment with phosphatase. Most of the enzyme of extracts prepared from livers of fed animals must, therefore, be in the active (dephosphorylated) form.

Inactivation of the liver enzyme by ATP was incomplete (Fig. 1) because of depletion of ATP by ATPase activity in the extracts. Indeed, very rapid destruction of ATP prevented ATP inactivation of the complex at 37° C (Fig. 2). This conclusion was confirmed by measurements of ATP concentrations and with the finding that an ATP-regenerating system (10 mM creatine phosphate plus 20

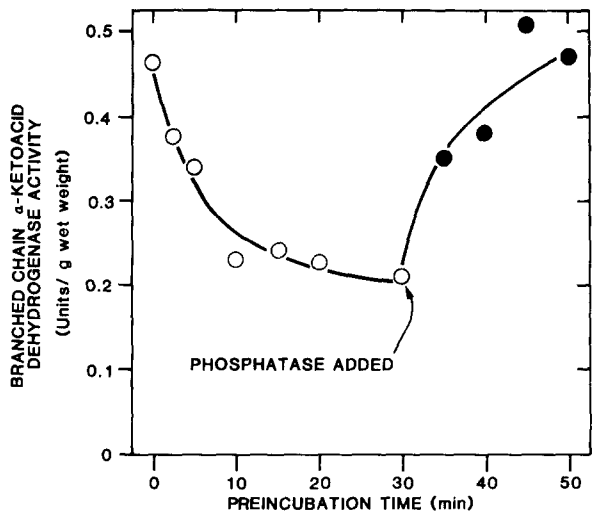

Figure 1. ATP inactivation of branched chain α-ketoacid dehydrogenase in a crude extract of liver followed by reactivation with the broad specificity protein phosphatase. The extract (100 µl) was incubated in a total volume of 250 µl containing 30 mM KPi, pH 7.5, 0.2 mM ATP, 0.1 mM $MgCl_2$, and 0.5 mM dithiothreitol at 20° C. Aliquots (25 µl) were removed at the indicated times and assayed for branched chain α-ketoacid dehydrogenase activity. Activation was carried out at 37° C by addition of 20 µl of phosphatase to 50 µl of incubation mixture as described in Materials and Methods.

units of creatine phosphokinase) brought about complete inactivation of the complex within 20 min (data not shown).

Virtually all of the enzyme from livers of fasted, high-protein fed, and diabetic rats was in the active state (Table 1). Protein starvation was the only condition found to produce significant effects on the activity state of the enzyme. The total amount of the enzyme was greatly reduced with only 33% of the remaining enzyme in the active state (Table 1). Although not nearly as dramatic, less total enzyme activity was also found with the high-dose streptozotocin-treated rats (Table 1).

Activity State of Branched Chain α-Ketoacid Dehydrogenase in Kidney. The branched chain α-ketoacid dehydrogenase activity of kidney extracts decreased on incubation at 37° C (data not shown), making it more difficult to ascertain the % active form than with liver or heart. Much of the problem was alleviated by using bovine serum as protease inhibitor. In order to correct for the loss of activity during incubation, the activity of phosphatase-treated samples was divided by the % activity remaining after incubation of the extracts without

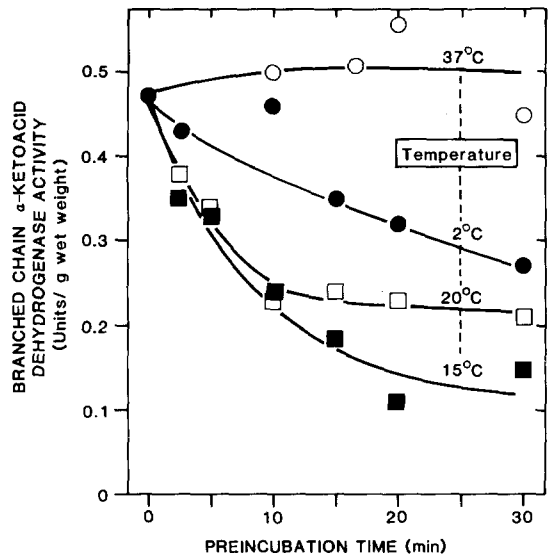

Figure 2. ATP inactivation of branched chain α-ketoacid dehydrogenase in a crude extract of liver at different incubation temperatures. ATP inactivation was carried out at the indicated temperatures as described in Figure 1.

phosphatase. With this correction, 70 to 80% of the kidney enzyme was in the active form in fed, fasted, diabetic and high-protein fed rats (Table 1). The kidney enzyme, like the liver enzyme, showed a substantial decrease in the % active form in protein-starved rats (Table 1). Unlike the liver, total enzyme activity was not decreased in the diabetic or low-protein fed animals. Increased total enzyme activity was observed in high-protein fed rats.

DISCUSSION. The results show that the branched chain α-ketoacid dehydrogenase complex is subject to in vivo regulation by covalent modification. The % active form of the enzyme clearly changes in three different tissues in response to change in nutritional and hormonal state.

Previous studies from this laboratory have shown conditions which change the capacity of the perfused heart to oxidize [1-^{14}C]leucine also cause a corresponding change in the proportion of the enzyme in the active state (5,6,10). The present study is the first to demonstrate that fasting and diabetes decrease the proportion of the heart enzyme in the active state.

The liver enzyme was nearly 100% active in 4 different physiological states. This apparent full capacity of branched chain α-ketoacid dehydrogenase

activity was not due to an intrinsic insensitivity of the liver enzyme to phosphatase since the complex could be inactivated with ATP and subsequently activated with phosphatase. Furthermore, rats subjected to an 8% protein diet for 12 days contained liver branched chain α-ketoacid dehydrogenase with a very small initial activity which could be dephosphorylated by phosphatase to greater activity. Reduced capacity to metabolize branched chain amino acids (11,12) and decreased branched chain α-ketoacid dehydrogenase activity (13,14) have been documented previously for the liver of protein-starved rats. The decrease in % active form of the liver enzyme in protein-starved rats is a new observation. Diurnal variation in activity has been reported for the liver enzyme (13). Covalent modification of the enzyme is the most likely explanation for these short-term changes in enzyme activity.

Since the branched chain α-ketoacid dehydrogenase complex is subject to regulation by cyclic phosphorylation-dephosphorylation, it is important to obtain tissue samples by the freeze-clamp technique and to extract the complex quantitatively under conditions that will protect the phosphorylation state of the enzyme. The extraction mixture used in this study contained: (a) Triton X-100 for quantitative extraction of the enzyme; (b) protease inhibitors to protect against proteolysis; (c) α-ketoisocaproate to inhibit the kinase; and (d) EDTA to protect against the phosphatase. The results reported here do not agree with previous estimates of the proportion of the liver enzyme in the active form (15), perhaps because mitochondria were isolated from the tissue in the previous study, a different extraction procedure for the enzyme was used, and no precautions were taken to preserve the phosphorylation state. Whether the phosphorylation state of the complex changes during the isolation of mitochondria is not known. Under the right conditions, however, ATP inactivation of the complex in liver extracts can occur at 2° C (Fig. 2), making it apparent that low temperature is not adequate protection against kinase activity.

The importance of the above conditions as well as the use of the phosphatase to estimate the maximum branched chain α-ketoacid dehydrogenase activity is reflected by the comparison of activities previously reported. For example,

heart, liver, and kidney branched chain α-ketoacid dehydrogenase activities were reported (16) as 9.2, 502, and 145 (nmol/min/g wet wt), respectively. These values, with the exception of liver, are considerably lower than reported in this study and underestimate the total capacity of branched chain α-ketoacid metabolism by heart and kidney by about 30- and 2-fold, respectively.

The nutritional and hormonal alterations used in this study were selected because of their known effects on branched chain amino acid metabolism as reflected in blood concentrations of branched chain amino and α-ketoacids (17-21). The importance of each tissue as well as the mechanisms involved in these alterations are not clearly understood. However, studies of the importance of covalent modification should further the understanding of the role of branched chain α-ketoacid dehydrogenase in branched chain amino acid metabolism in each tissue. For example, the reduced % active form of the liver enzyme seen with low-protein diet and of the heart enzyme seen with streptozotocin treatment are consistent with the elevated tissue level of α-ketoisocaproate seen in each tissue under these conditions (20). It is apparent that regulation of branched chain α-ketoacid dehydrogenase is tissue specific since with fed, fasted, and diabetic rats the liver enzyme is completely active while this is not true for the heart and kidney enzymes.

Experiments are currently in progress to define the proportion of the enzyme in the active state in muscle and adipose tissue.

ACKNOWLEDGMENTS. This work was supported in part by grants from the U. S. Public Health Service (AM19259), the Diabetes Research and Training Center of Indiana University School of Medicine (AM20542), Indiana Affiliate of American Heart Association, and the Grace M. Showalter Residuary Trust. SEG received a student fellowship from the American Diabetes Association (Indiana Affiliate). GAC is an Established Investigator of the American Heart Association.

REFERENCES

1. Fatania, H.R., Lau, K.S., and Randle, P.J. (1981) FEBS Lett. 132, 285-288.
2. Odessey, R. (1982) Biochem. J. 204, 353-356.
3. Hughes, W.A., and Halestrap, A.P. (1981) Biochem. J. 196, 459-469.
4. Paxton, R., and Harris, R.A. (1982) J. Biol. Chem. 257, 14433-14439.
5. Harris, R.A., Paxton, R., and Parker, R.A. (1982) Biochem. Biophys. Res. Commun. 107, 149-150.
6. Harris, R.A., Paxton, R., and DePaoli-Roach, A.A. (1982) J. Biol. Chem. 257, 13915-13918.
7. Schein, P.S., Alberti, K.G.M.M., and Williamson, D.H. (1971) Endocrinology 89, 827-834.

8. Williamson, D.A., Mellanby, J., and Krebs, H.A. (1962) Biochem. J. 82, 90-96.
9. Parker, P.J., and Randle, P.J. (1980) FEBS Lett. 112, 186-190.
10. Sans, R.M., Jolly, W.W., and Harris, R.A. (1980) Arch. Biochem. Biophys. 200, 336-345.
11. McFarlane, I.G., and von Holt, C. (1969) Biochem. J. 111, 557-563.
12. Sketcher, R.D., and James, W.P.T. (1974) Brit. J. Nutr. 32, 615-623.
13. Wohlheuter, R.M., and Harper, A.E. (1970) J. Biol. Chem. 245, 2391-2401.
14. Khatra, B.S., Chawla, R.K., Wadsworth, A.D., and Rudman, D. (1977) J. Nutr. 107, 1528-1536.
15. Paul, H.S., and Adibi, S.A. (1982) J. Biol. Chem. 257, 4875-4881.
16. Shinnick, F.L., Jr., and Harper, A.E. (1977) J. Nutr. 107, 887-895.
17. Felig, P., Martiss, E., Ohman, J., and Cahill, G.F. (1970) Diabetes 19, 727-729.
18. Adibi, S.A. (1976) Metabolism 25, 1287-1302.
19. Livesey, G., and Lund, P. (1980) Biochem. J. 188, 705-713.
20. Hutson, S.M., and Harper, A.E. (1981) Am. J. Clin. Nutr. 34, 173-183.
21. Vannini, P., Marchesini, G., Forlani, G., Argiolini, A., Ciavarella, A., Zoli, M., and Pisi, E. (1982) Diabetologia 22, 217-219.