Evidence for the Regulation of the Branched Chain α-Keto Acid Dehydrogenase Multienzyme Complex by a Phosphorylation/Dephosphorylation Mechanism†

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ABSTRACT: The regulation of the branched chain α-keto acid dehydrogenase complex by covalent modification was investigated in rat liver mitochondria. Depletion of intramitochondrial calcium and magnesium caused an inactivation of the branched chain α-keto acid dehydrogenase complex. Following inactivation of the branched chain complex, addition of calcium or magnesium ions separately to incubations of mitochondria only partially reactivated the enzyme complex. However, simultaneous addition of calcium and magnesium activated the branched chain enzyme complex rapidly and nearly completely. Mitochondrial incubations were performed in the presence of [^{32}P]phosphate under conditions known to activate or to inactivate the branched chain α-keto acid dehydrogenase complex. Evidence demonstrating that [^{32}P]-phosphate was incorporated into two major protein bands separated in sodium dodecyl sulfate–polyacrylamide gels of the mitochondrial incubations is presented. Migration of the labeled mitochondrial protein bands in the gel system corresponded exactly to the migration of the α subunit of the purified heart-derived pyruvate dehydrogenase (decarboxylase, E1) and the α subunit of the purified kidney-derived branched chain α-keto acid dehydrogenase (decarboxylase, E1). Furthermore, when the measured activity of the branched chain complex was minimized, the amount of [^{32}P]phosphate incorporated into the α chain of the branched chain enzyme was maximal. Conversely, incubation conditions which activated maximally the enzyme complex minimized the [^{32}P]phosphate incorporation into the α subunit of the branched chain dehydrogenase.

The branched chain α-keto acids α-ketoisocaproate, α-ketoisovalerate, and α-keto-β-methylvalerate are oxidized by the branched chain α-keto acid dehydrogenase multienzyme complex to carbon dioxide, reduced nicotinamide adenine dinucleotide (NADH), and the respective acyl coenzyme A (acyl-CoA) derivatives (Wohlheuter & Harper, 1970; Adibi, 1976; Parker & Randle, 1978a,b). The catalytic mechanism for this reaction is very similar to the pyruvate and the α-ketoglutarate dehydrogenase complexes (Reed & Oliver, 1968; Koike & Koike, 1976) and uses CoASH, NAD^+, and thiamine pyrophosphate as the requisite coenzymes in the catalytic mechanism of this reaction. The branched chain α-keto acid dehydrogenase complex has been implicated as the rate-limiting step in the catabolism of leucine, isoleucine, and valine and, therefore, has the potential to regulate energy generation, ketone body synthesis, and glucose production from these amino acids (Odessey & Goldberg, 1972; Buse et al., 1975). The regulation of branched chain α-keto acid metabolism may occur at several levels. First, both α-ketoisocaproate and α-ketoisovalerate are transported across the mitochondrial membrane via the monocarboxylate transporter (Patel et al., 1980). Since the branched chain α-keto acid dehydrogenase complex is located in the mitochondrial matrix (Bremer & Davis, 1978; Van Hinsberg et al., 1978, 1979; Patel et al., 1980), any modulator of the monocarboxylate transporter would affect the intramitochondrial concentration of the branched chain α-keto acids and, thus, the metabolic flux through the branched chain enzyme complex (Patel et al., 1981a; T. B. Patel, L. L. Barron, and M. S. Olson, unpublished experiments). Second, products of the branched chain dehydrogenase reaction, NADH and branched chain acyl-CoA derivatives, inhibit the enzyme complex competitively (Pettit et al., 1978; Parker & Randle, 1978b).

Indeed, in perfused rat heart and in liver preparations, inhibition of the metabolic flux through the branched chain dehydrogenase complex was observed under perfusion conditions which elevated the mitochondrial NADH/NAD^+ or acyl-CoA/CoASH ratios (Buffington et al., 1979; Patel et al., 1981b; T. B. Patel, L. L. Barron, and M. S. Olson, unpublished experiments).

Third, the branched chain α-keto acid dehydrogenase complex in the perfused rat heart is converted to a stable, inhibited state in the presence of pyruvate by a mechanism which remains to be fully elucidated (Waymack et al., 1980). Branched chain α-keto acids protect the enzyme complex from the pyruvate-mediated inactivation process.

Finally, it has been suggested that the branched chain dehydrogenase complex is regulated by a phosphorylation/dephosphorylation mechanism (Johnson & Connelly, 1972; Buse et al., 1972; Gubler & Malquist, 1979). While experimental evidence from several laboratories is consistent with this contention, a conclusive documentation of a protein kinase mediated inactivation of the branched chain enzyme complex has not been provided. A reduction of the assayable branched chain dehydrogenase activity upon treatment with ATP has been reported using mitochondria derived from liver (Johnson & Connelly, 1972; Gubler & Malquist, 1979; Lau et al., 1981), skeletal muscle (Goldberg & Chang, 1978; Odessey & Goldberg, 1979), heart (Parker & Randle, 1978c, 1980; Buffington et al., 1981; C. K. Buffington, T. B. Patel, P. P. Waymack, and M. S. Olson, unpublished experiments), and kidney (Odessey, 1980a,b; Lau et al., 1981). Each of these reports contends that an elevated mitochondrial ATP/ADP ratio results in the phosphorylation of the branched chain complex by a protein kinase which inactivates the enzyme complex. Further, it has been suggested that a phosphoprotein phosphatase reactivates the phosphoenzyme by cleaving the

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phosphoryl group from the inactive enzyme. However, neither the putative protein kinase nor the phosphoprotein phosphatase has been described as of this date [e.g., see Pettit et al. (1978) and Danner et al. (1979)]. In most of these studies cited above, the inactivation of the branched chain complex was assumed to occur by a mechanism analogous to that documented for the pyruvate dehydrogenase complex [e.g., see Olson et al. (1981) for a discussion].

Preliminary reports from three laboratories have indicated that [^{32}P]phosphate may be incorporated into the branched chain α-keto acid dehydrogenase in mitochondrial incubations (Odessey, 1980b; Lau et al., 1981; Hughes & Halestrap, 1981). However, definitive documentation that the phosphorylated species which migrates in sodium dodecyl sulfate–polyacrylamide gels as a 46 000–48 000 molecular weight protein was the α subunit of the branched chain dehydrogenase was not demonstrated rigorously in these reports. There has been only one preliminary report indicating a correlation between changes in [^{32}P]phosphate incorporation into the putative α subunit of the branched chain dehydrogenase and changes in the measured branched chain α-keto acid dehydrogenase activity in the purified complex from bovine kidney (Fatania et al., 1981). However, such evidence in an intact metabolic system has not been provided.

The present study was conducted to ascertain whether (a) the activity of the branched chain α-keto acid dehydrogenase can be increased in liver mitochondria under incubation conditions known to facilitate protein kinase activity on one hand and phosphoprotein phosphatase activity on the other and (b) the incorporation of [^{32}P]phosphate into a molecular species which comigrates with the purified α subunit of the branched chain dehydrogenase (decarboxylase) on sodium dodecyl sulfate ($NaDodSO_4$) gels can be correlated with the assayable enzymatic activity of the mitochondrial branched chain α-keto acid dehydrogenase.

Materials and Methods

Mitochondria were isolated from the livers of male Sprague-Dawley rats (180–200 g body weight) by using the method of Schneider & Hogeboom (1950) with only minor modifications. The isolation medium contained mannitol (75 mM), sucrose (225 mM), and ethylenediaminetetraacetic acid (EDTA) (0.1 mM). The biuret procedure described by Gornall et al. (1949) was utilized for mitochondrial protein determinations. Mitochondrial preparations used in these experiments did not oxidize exogenous NADH and exhibited respiratory control ratios of 4 or greater with pyruvate (5 mM) and L-malate (2.5 mM) as cosubstrates. Mitochondrial incubations were performed in a glass reaction chamber maintained at 30 °C. The incubation medium employed was saturated with oxygen and contained the following components: potassium chloride, 130 mM; Tris-HCl, 20 mM; and potassium phosphate, 5 mM, at pH 7.2 (Walajtys-Rode et al., 1974). Mitochondria (approximately 4 mg of protein) were incubated in this reaction medium (final volume 2.0 mL) under the various conditions noted in the individual experiments. Samples (0.2 mL) of the incubation were withdrawn at various time points, and the branched chain α-keto acid dehydrogenase activity was assayed by monitoring the stoichiometric increase in NADH production spectrophotometrically at 340 nm. The branched chain dehydrogenase assay mixture contained the following: Tris-HCl, 100 mM, pH 7.8; NAD^+, 10 mM; thiamine pyrophosphate, 2 mM; dithiothreitol, 1 mM; sodium fluoride, 25 mM; coenzyme A, 1 mM; magnesium chloride, 5 mM; rotenone, 20 μM; Triton X-100, 0.1% (v/v); and either α-ketoisovalerate or α-ketoisocaproate, 5 mM. Assays were performed at 25 °C, and the incubation contained approximately 0.4 mg of mitochondrial protein.

Incorporation of [^{32}P]phosphate into mitochondrial protein was monitored in the following way. Mitochondrial incubations (0.5 mL final volume) were performed exactly as described above for the enzyme activity determinations except that the incubation medium contained [^{32}P]phosphate at a specific radioactivity of 200 dpm/pmol. The incubations were terminated by addition of 0.5 mL of 10% trichloroacetic acid. The resulting precipitate was washed 5 times with 3 mL (per wash) of 10% trichloroacetic acid, and the final precipitate was dissolved in 0.4 mL of the sample medium described by Laemmli (1970). Mitochondrial samples (approximately 100 μg of protein) were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis in 14 × 10.5 × 0.3 cm slabs (10% polyacrylamide) at 100 V. Purified pyruvate dehydrogenase (E1) from bovine heart and partially purified branched chain α-keto acid dehydrogenase (E1) from bovine kidney and appropriate molecular weight standards (noted in the figure legend) were employed as molecular weight markers to which the phosphorylated mitochondrial proteins were compared. The running buffer (Tris–glycine) and the gels were prepared as described by Laemmli (1970). Autoradiographs of the gels were prepared by using Agfa Gevaert Curix RP1 X-ray film. The relative amount of protein applied to each gel track was estimated by scanning the Coomassie Blue stained gels with a microdensitometer. Subsequent densitometer scans of the [^{32}P]phosphate-containing protein bands on the autoradiograph were corrected for any significant difference in the protein in the individual bands.

The sodium salts of α-ketoisovalerate and α-ketoisocaproate were purchased from Sigma Chemical Co. (St. Louis, MO). Coenzyme A, NAD^+, thiamine pyrophosphate, and dithiothreitol were obtained from P-L Biochemicals (Milwaukee, WI). The ionophore A23187 was obtained from Calbiochem-Behring Corp. (La Jolla, CA). [^{32}P]Phosphate was purchased from ICN Chemicals and Radioisotope Division (Irvine, CA). Samples of the purified pyruvate dehydrogenase (E1) and the partially purified branched chain α-keto acid dehydrogenase (E1) were the generous gifts of Drs. Lester J. Reed and Flora Pettit of The University of Texas (Austin, TX). The uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) was obtained from Dr. P. G. Heytler of E. I. du Pont de Nemours and Co., Inc. Magnesium chloride and calcium chloride were obtained from Mallinckrodt Chemical Works (St. Louis, MO) and Fisher Scientific Co. (Fairlawn, NJ), respectively, and were of analytical grade. All other chemicals were of the highest purity available from commercial suppliers.

Results

There is ample suggestive evidence in the literature that the branched chain α-keto acid dehydrogenase complex may be regulated in a fashion similar to the well-characterized kinase/phosphatase-mediated covalent modification of the pyruvate dehydrogenase complex in various tissues. The initial focus of the present study was to ascertain whether the branched chain α-keto acid dehydrogenase activity in liver mitochondria might be sensitive to metabolic manipulations known to alter the pyruvate dehydrogenase kinase/phosphatase equilibrium in liver mitochondria [e.g., see Walajtys-Rode et al. (1974)] by affecting the activity of the pyruvate dehydrogenase phosphatase because of its sensitivity to the metal cations calcium and magnesium (Schuster & Olson, 1974).

The experiment shown in Figure 1 demonstrates that the branched chain α-keto acid dehydrogenase activity in isolated

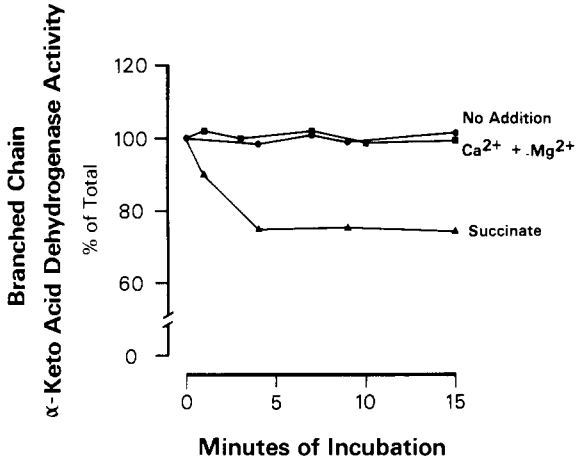

FIGURE 1: Effects of divalent metal cations and succinate on the activity of the branched chain α-keto acid dehydrogenase in isolated rat liver mitochondria. Mitochondria were incubated at 30 °C (a) with no additions (●), (b) in the presence of calcium chloride, 2 mM, and magnesium chloride, 5 mM (■), and (c) with succinate (state 4) (▲). Samples (0.2 mL) of the incubation mixture were withdrawn at the time points indicated, and the activity of the branched chain α-keto acid dehydrogenase was assayed as described under Materials and Methods.

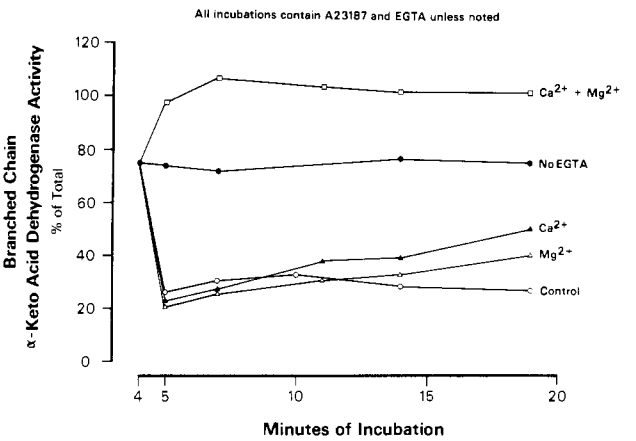

FIGURE 2: Effects of A23187, EGTA, calcium, and magnesium on the branched chain α-keto acid dehydrogenase activity in rat liver mitochondria. Mitochondrial incubations and the branched chain dehydrogenase assay were performed on samples of the various incubations as indicated under Materials and Methods. The concentrations of the various components were as follows: A23187, 20 μM; EGTA, 0.5 mM; calcium chloride, 2 mM; magnesium chloride, 5 mM.

rat liver mitochondria is stable to incubation for a 15-min interval. This result contrasts with findings using cardiac-derived mitochondria in which the enzyme complex is activated substantially by substrate-free incubation (Parker & Randle, 1980; Buffington et al., 1981). In fact, this observation confirms the finding of Lau et al. (1981) that the branched chain enzyme complex in freshly isolated liver mitochondrial preparations is in a fully active state. Addition of either calcium or magnesium to the incubations had no measurable effect on the activity of the branched chain complex. Pyruvate dehydrogenase, on the other hand, has been shown to be activated by the inclusion of calcium and magnesium in mitochondrial incubations [e.g., see Schuster & Olson (1974) and Walajtys-Rode et al. (1974)]. Additionally, magnesium ions have been reported to increase the measurable branched chain dehydrogenase activity in skeletal muscle and heart mitochondria (Odessey & Goldberg, 1979; Parker & Randle, 1980; Odessey, 1980a). When liver mitochondria were incubated in state 4 (Chance & Williams, 1956) with succinate (5 mM) as the respiratory substrate, a time-dependent decrease in the branched chain dehydrogenase activity was observed (Figure 1). The activity of the enzyme complex (initial activity 9.9 $nmol \cdot min^{-1} \cdot mg^{-1}$) decreased to a steady-state level approximately 75% of the control value within 4 min after the initiation of the incubation with succinate addition.

For facilitation of the study of the effects of the divalent metal cations, calcium and magnesium, on the regulation of the branched chain dehydrogenase, the experiment shown in Figure 2 was performed. Liver mitochondria were incubated under state 4 conditions for 4 min with succinate as the respiratory substrate, and as demonstrated in Figure 1, the branched chain dehydrogenase activity declined to about 75% of the control value. At this point (indicated as the time zero point in Figure 2), various additions were made to the mitochondrial incubation. The activity of the branched chain dehydrogenase was measured for an additional 15-min interval and plotted as a percentage of the maximum measurable enzyme activity, i.e., 9.9 $nmol \cdot min^{-1} \cdot mg^{-1}$, as described under Materials and Methods. Incubation of the mitochondria in the presence of A23187 (20 μM) resulted in no change in the measured activity of the branched chain dehydrogenase. However, addition of A23187 plus ethylene glycol bis(β-aminoethyl)ether-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (0.5 mM) resulted in a condition during which (a) the endogenous divalent metal cations were partially lost [e.g., see Walajtys-Rode et al. (1974)], (b) the pyruvate dehydrogenase reaction was inactivated, and (c) a large decline occurred in the branched chain dehydrogenase activity to a level only 26% of the control, uninhibited level within 1 min. This inhibited activity was maintained throughout the remainder of the incubation period. Addition of calcium or magnesium separately to the liver mitochondria incubated in state 4 with A23187 and EGTA present did not affect the decline in the branched chain dehydrogenase activity observed in the initial minute of these incubations. Addition of calcium or magnesium separately to mitochondria incubated with A23187 plus EGTA resulted in a gradual but incomplete recovery of the branched chain dehydrogenase activity following the initial inhibitory phase. When calcium and magnesium were added together (with A23187 and EGTA), a condition which increases markedly the matrix calcium and magnesium level (Walajtys-Rode et al., 1974), there occurred an activation of the branched chain dehydrogenase activity to a level equivalent to the control value prior to substrate addition (e.g., state 4). Hence, both divalent metal cations appeared to be necessary for the expression of the full activity of the branched chain complex.

For further investigation of the activation of the rat liver branched chain dehydrogenase complex by calcium and magnesium, the experiments represented in Figure 3 were conducted. Rat liver mitochondria were incubated for 4 min in state 4 with succinate (5 mM) as the respiratory substrate, and the branched chain dehydrogenase activity declined to nearly 71% of the original activity. At the point designated time 0 in Figure 3, EGTA and A23187 were added to the incubation, and the activity was measured 1 and 5 min later. The mitochondrial incubation was centrifuged at 7700*g* for 10 min, and the mitochondrial pellet was resuspended in the original incubation medium containing 25 mM sodium fluoride and centrifuged again. The mitochondrial pellet was washed twice with incubation medium to remove any residual sodium fluoride. Measurement of the branched chain dehydrogenase activity indicated the absence of any detectable enzyme activity. If these mitochondria were incubated in the presence of sodium fluoride, the activity of the branched chain complex

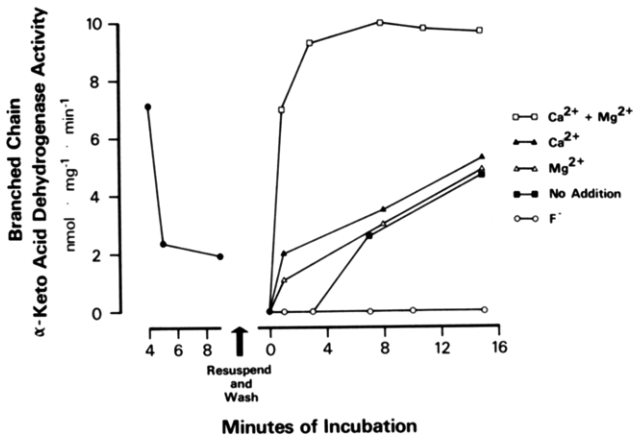

FIGURE 3: Inactivation and reactivation of the branched chain α-keto acid dehydrogenase in rat liver mitochondria. Mitochondria were incubated with succinate (5 mM) (state 4) for 4 min. A23187 (20 μM) and EGTA (0.5 mM) were added, and samples were removed for estimation of the activity of the branched chain dehydrogenase. The mitochondrial incubation was centrifuged, and the mitochondria were resuspended and washed as described in the text. The mitochondria containing inactivated branched chain dehydrogenase activity were incubated in the presence of the various components noted at the following final concentrations: calcium chloride, 2 mM; magnesium chloride, 5 mM; sodium fluoride, 25 mM.

remained at a minimal value. Mitochondria reincubated without further addition or in the presence of calcium or magnesium separately caused a modest (e.g., 50% after 15 min) reactivation of the branched chain dehydrogenase activity. Incubation of the liver mitochondria following this inactivation treatment in the presence of both divalent metal cations caused a rapid and extensive (i.e., to 100% of the original activity) reactivation of the branched chain dehydrogenase activity during the next 4 min of incubation. The experiment illustrated in Figure 3 indicates that the branched chain dehydrogenase not only can be inactivated by removal of calcium and magnesium from the mitochondria (i.e., using A23187 plus EGTA) but also can be reactivated rapidly by replenishing both divalent metal cations.

The possibility that the branched chain α-keto acid dehydrogenase complex may be regulated by a covalent modification mechanism, e.g., phosphorylation, was investigated in this liver mitochondrial system. Mitochondrial incubations were performed in the presence of [^{32}P]phosphate in order to ascertain whether [^{32}P]phosphate might be incorporated into the branched chain dehydrogenase complex during incubation of the mitochondria under conditions in which we have measured significant alterations of the enzymatic activity of the enzyme complex. Five metabolic conditions were selected on the basis of the information concerning the changes in the enzymatic activity of the branched chain complex observed in Figures 1–3 (see legends for Figures 4 and 5). The mitochondrial incubations were processed as described under Materials and Methods. Figure 4 shows a NaDodSO$_4$–polyacrylamide gel of the array of mitochondrial proteins observed in the five different incubations which were performed. Flanking the lanes in which the mitochondrial proteins were run (lanes IV–VIII) are samples of (a) purified bovine heart pyruvate dehydrogenase (decarboxylase, E1) (lanes I and IX), (b) bovine kidney branched chain α-keto acid dehydrogenase (decarboxylase, E1) (lanes III and X), and (c) various molecular weight standards (lane II) for comparison. The α subunits of the pyruvate dehydrogenase and the branched chain α-keto acid dehydrogenase components of the respective multienzyme complexes were separated according to their respective molecular weights (e.g., approximately

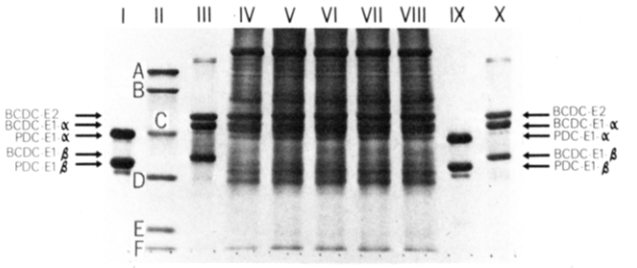

FIGURE 4: NaDodSO$_4$–polyacrylamide gel electrophoresis of mitochondrial proteins. NaDodSO$_4$–polyacrylamide gels were performed as described under Materials and Methods. Reference proteins include the bovine heart pyruvate dehydrogenase (decarboxylase, E1) (lanes I and IX), the bovine kidney branched chain α-keto acid dehydrogenase (decarboxylase, E1) (lanes III and X) (this sample contains some contaminating dihydrolipoyl transacylase, E2), appropriate molecular weight standards (lane II), and mitochondrial extracts from various incubations (lanes IV–VIII). The incubation conditions of the mitochondrial samples are listed in the legend to Figure 5. Molecular weight standards employed were (A) phosphorylase *b* (92 500), (B) bovine serum albumin (66 200), (C) ovalbumin (45 000), (D) carbonic anhydrase (31 000), (E) soybean trypsin inhibitor (21 500), and (F) lysozyme (14 400).

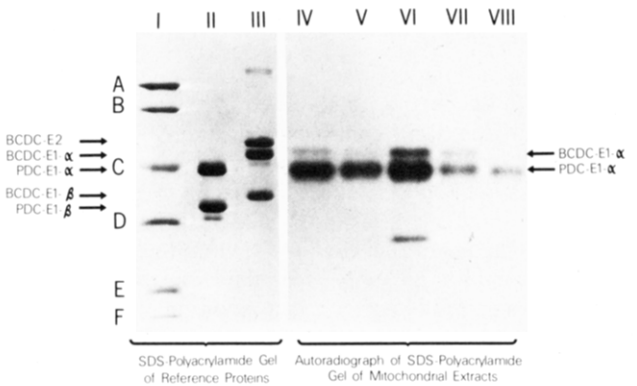

FIGURE 5: Autoradiographic analysis of a NaDodSO$_4$–polyacrylamide gel of various mitochondrial extracts. The mitochondrial extracts were electrophoresed in a NaDodSO$_4$–polyacrylamide gel (lanes IV–VIII), and an autoradiograph was prepared as described under Materials and Methods for comparison with the migration of reference proteins in similar gels (lanes I–III). Mitochondrial incubations were performed as defined under Materials and Methods under the following conditions: lane IV, no addition, 10 min; lane V, succinate (5 mM) (state 4), 4 min; lane VI, succinate, 4 min, then EGTA (0.5 mM) plus A23187 (20 μM), 10 min; lane VII, succinate, 4 min, then EGTA plus A23187, calcium chloride (2 mM), and magnesium chloride (5 mM), 10 min; lane VIII, succinate, 4 min, then uncoupler (FCCP) (5 μM), magnesium chloride (1 mM), sodium fluoride (25 mM), and ATP (4 mM), 10 min.

45 000 for the α chain of pyruvate dehydrogenase and approximately 48 500 for the α chain of the branched chain dehydrogenase) as estimated from the migration of the reference proteins in this gel electrophoresis system. The estimated molecular weights for the respective α chains from our experiments were similar to those reported by Pettit et al. (1978). The autoradiograph of the NaDodSO$_4$–polyacrylamide gel of the mitochondrial incubations shown in Figure 5 revealed that two major protein bands incorporated the [^{32}P]phosphate label. These two labeled bands coincided nearly exactly with the resolved α subunits of the pyruvate and branched chain dehydrogenases derived from the bovine heart and kidney complexes, respectively. For comparison of the effect of the various incubation conditions on the incorporation of [^{32}P]phosphate into the α subunits of the two dehydrogenases, the protein bands, stained with Coomassie Blue, were scanned with a densitometer to estimate the relative amount of protein contained in each band of the individual

Table I: Comparison of Relative ^{32}P Labeling of Branched Chain α-Keto Acid Dehydrogenase and Pyruvate Dehydrogenase in Rat Liver Mitochondria[a]

incubation conditions	branched chain dehydrogenase		pyruvate dehydrogenase
	α-subunit area (mm^2)	enzymatic activity ($nmol \cdot min^{-1} \cdot mg^{-1}$)	α-subunit area (mm^2)
I, no addition, 10 min	52	9.9	465
II, succinate, 5 mM, 4 min	82	7.3	465
III, succinate, 5 mM, 4 min; then EGTA, 0.5 mM, and A23187, 20 μM, 10 min	306	1.9	819
IV, succinate, 5 mM, 4 min; then EGTA, 0.5 mM; A23187, 20 μM; $CaCl_2$, 2 mM; and $MgCl_2$, 5 mM, 10 min	120	9.7	292
V, succinate, 5 mM, 4 min; then FCCP, 5 μM; $MgCl_2$, 1 mM; NaF, 25 mM; and ATP, 4 mM, 10 min	20	9.5	412

[a] Bands in the autoradiograph of the $NaDodSO_4$–polyacrylamide gels of mitochondrial extracts which correponded to the α subunit of the pyruvate and branched chain dehydrogenases were scanned with a densitometer. Areas under the respective peaks were measured in mm^2 and were corrected for protein in each peak as described under Materials and Methods.

gel tracks. Unless an attempt was made to normalize the amount of protein appearing in the various bands, any attempted correlation of the [^{32}P]phosphate incorporation into the branched chain α subunit and the enzymatic activity present in the mitochondrial incubation would be less than valid. Hence, the autoradiographs were scanned, and the area under each peak was normalized for the protein in each band.

Table I indicates that the greatest incorporation of the [^{32}P]phosphate level into the α subunits of the two dehydrogenases occurred in the incubation to which A23187 and EGTA were added. These same incubation conditions resulted in the lowest measured activity of the branched chain complex (Table I) and are the conditions which result in the depletion of the metal cation content of the mitochondrial matrix. Relatively little [^{32}P]phosphate was incorporated into the α chain of the branched chain complex when the mitochondria were incubated without additions or in mitochondria to which uncoupler, magnesium chloride, sodium fluoride, and ATP were added, both situations in which the enzymatic activity of the branched chain complex was high, e.g., 9.5 and 9.8 $mmol \cdot min^{-1} \cdot mg^{-1}$, respectively. Addition of calcium and magnesium to the state 4 mitochondria incubated in the presence of A23187 and EDTA diminished the incorporation of [^{32}P]phosphate into the branched chain complex and resulted in an elevated enzymatic activity. It should be pointed out that the extent of [^{32}P]phosphate labeling of the α chain of the branched chain dehydrogenase was more than might have been predicted on the basis of the amount of enzymatic activity observed when calcium and magnesium were added to the incubation containing A23187 and EGTA.

Discussion

While a number of reports have indicated that the branched chain α-keto acid dehydrogenase complex may be regulated by a protein kinase/phosphatase-mediated phosphorylation/dephosphorylation mechanism analogous to but separate from the well-characterized pyruvate dehydrogenase system, unequivocal proof of such a suggestion has not been presented. As pointed out above, it is clear that the branched chain dehydrogenase activity of various types of isolated mitochondria can be inhibited (inactivated?) in an ATP-, magnesium-, and time-dependent fashion. Further, there are preliminary reports that [^{32}P]phosphate may be incorporated into the α chains of the branched chain α-keto acid dehydrogenase (decarboxylase, E1) in various tissues [e.g., see Odessey (1980b), Lau et al. (1981), and Hughes & Halestrap (1981)]. However, several concerns must be addressed relative to these preliminary studies and may be generalized as the following: (a) rigorous experimental evidence of the identity of the [^{32}P]phosphate-labeled proteins observed in the chromatographic or electrophoretic systems employed was not provided; (b) correlation of the extent of [^{32}P]phosphate incorporation into the putative branched chain dehydrogenase α chains with changes in the enzymatic activity of the branched chain enzyme complex has been highly inconsistent; and (c) definition and characterization of the putative protein kinase and phosphatase responsible for the phorphorylation/dephosphorylation inactivation/activation cycles of the enzyme complex have not been forthcoming. The present study was initiated to develop experimental evidence which may allow us to evaluate the possibility of a covalent modification mediated regulatory mechanism for the branched chain dehydrogenase complex.

Unlike the situation in cardiac tissue (Waymack et al., 1980), the branched chain α-keto acid dehydrogenase in freshly isolated rat liver mitochondria appears to exist in an enzymatically active form. Addition of A23187 and EGTA to state 4 liver mitochondria, metabolic conditions which should lead to the depletion of mitochondrial divalent metal cations, caused a rapid and extensive inactivation of the branched chain dehydrogenase activity. Inclusion of the divalent metal cations calcium and magnesium individually in such experiments did not result in any major change in the activity of the branched chain complex. However, adding both calcium and magnesium to the incubation medium resulted in a substantial activation of the enzyme complex. These findings may be interpreted to mean that the divalent metal cations are necessary for the proper function of a phosphoprotein phosphatase which can activate an inactivated (phosphorylated) branched chain dehydrogenase complex in an analogous fashion to the effects of these same metal cations on the pyruvate dehydrogenase phosphatase. It should be pointed out that the branched chain dehydrogenase activity in rat heart mitochondria and extracts thereof may be activated by incubation in the presence of elevated magnesium concentrations (Odessey & Goldberg, 1979; Parker & Randle, 1980; Odessey, 1980a; Buffington et al., 1981).

The rat liver branched chain dehydrogenase complex was inactivated by nearly 25% under incubation conditions (i.e., state 4) which should result in increased intramitochondrial ratios of ATP/ADP and $NADH/NAD^+$ (Figure 1). In a similar fashion, the pyruvate dehydrogenase complex in liver mitochondria has been shown to be inactivated substantially merely by incubating the mitochondria in state 4 (Walajtys-

Rode et al., 1974; Batenburg & Olson, 1976). Hence, when the branched chain α-keto acid dehydrogenase is exposed to elevated intramitochondrial calcium and magnesium levels, the complex is activated, while depletion of these metal cations when the intramitochondrial ATP level is high (e.g., state 4) leads to an inactivation of the branched chain dehydrogenase.

Our ability to manipulate the assayable activity of the branched chain dehydrogenase in this mitochondrial system was exploited in our attempt to incorporate [^{32}P]phosphate into the enzyme complex. Evidence was presented suggesting that [^{32}P]phosphate was incorporated into two major protein bands observed in $NaDodSO_4$–polyacrylamide gels of our mitochondrial incubations. The migration of these labeled mitochondrial proteins in the gel system selected for this study corresponded exactly to those of the purified α subunits of the heart-derived pyruvate dehydrogenase (decarboxylase, E1) and the kidney-derived branched chain dehydrogenase (decarboxylase, E1) (Figure 5). Further, what we are referring to as the α chain of the branched chain dehydrogenase was maximally labeled with [^{32}P]phosphate when the measured enzymatic activity of the complex was minimized. The converse to this situation was also true; i.e., minimizing the [^{32}P]phosphate incorporation into the branched chain α chain maximally activated the enzyme complex derived from the mitochondrial incubation. Obtaining exact or stoichiometric correlations of the [^{32}P]phosphate content and the enzymatic activity state of the branched chain complex may depend upon determining the endogenous phosphate content of the branched chain complex prior to introduction of [^{32}P]phosphate into the enzyme.

In contrast to the report of Lau et al. (1981) indicating that the branched chain dehydrogenase activity in the liver may be inactivated by incubating the liver mitochondrial extracts in the presence of exogenous ATP, we were unable to either [^{32}P]phosphate label or inactivate the enzyme complex using exogenous ATP under any circumstance. Other studies in this laboratory indicate that the branched chain dehydrogenase complex in rat heart mitochondria is inactivated and labeled with [^{32}P]phosphate in the presence of ATP of exogenous origin.

In conclusion, our results are supportive of the concept that the branched chain α-keto acid dehydrogenase complex may be alternatively inactivated by an as yet undefined protein kinase and activated under conditions which should stimulate phosphoprotein phosphatase activity in a liver mitochondrial system. Further studies are required (a) to demonstrate conclusively the identity of the 48 500 molecular weight phosphorylated protein in these $NaDodSO_4$–polyacrylamide gels, (b) to define the regulatory properties of the putative protein kinase and phosphatase reactions, and (c) ultimately to isolate and to characterize the molecular properties of the specific protein kinase and phosphoprotein phosphatase operative on the branched chain α-keto acid dehydrogenase multienzyme complex.

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Oxidative Decarboxylation of Benzoate to Carbon Dioxide by Rat Liver Microsomes: A Probe for Oxygen Radical Production during Microsomal Electron Transfer†

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ABSTRACT: The oxidative decarboxylation of [7-^{14}C]benzoate has been used by others to evaluate ·OH production by phagocytes and during the xanthine–xanthine oxidase reaction. The current report concerns the use of benzoate as a probe for the detection of oxy radicals generated by another biological system, NADPH-dependent, microsomal electron transfer. $^{14}CO_2$ was produced from [7-^{14}C]benzoate by rat liver microsomes in a Chelex-treated incubation medium. The reaction was dependent upon an NADPH-generating system and intact microsomes. $^{14}CO_2$ was augmented nearly 10-fold in the presence of azide, an inhibitor of catalase, suggesting that H_2O_2 may serve as a precursor of ·OH. External addition of H_2O_2 also increased benzoate decarboxylation. The ·OH scavengers mannitol, dimethyl sulfoxide, 2-oxo-4-(methylthio)butyric acid, and ethanol inhibited $^{14}CO_2$ production. Kinetic studies suggested that the scavengers compete with benzoate for a common intermediate or site. Iron–ethylenediaminetetraacetic acid (EDTA), which catalyzes the decomposition of H_2O_2 resulting in the generation of ·OH (Fenton-type reaction), stimulated $^{14}CO_2$ production in a dose-dependent manner. Metyrapone and SKF-525A, typical inhibitors of mixed-function oxidase activity, did not significantly affect the decarboxylation of benzoate. Organic hydroperoxides such as cumene or *tert*-butyl hydroperoxide, which are capable of catalyzing the metabolism of certain drugs in the absence of NADPH, did not catalyze benzoate decarboxylation. These results disassociate the overall metabolism of benzoate from typical substrates of the mixed-function oxidase system and suggest that discrete pathways for microsomal metabolism of drugs and ·OH scavengers exist. The autoxidation of ascorbate by Fe^{3+} readily promoted benzoate decarboxylation in a manner consistent with a role for ·OH. These data suggest that $^{14}CO_2$ production from [7-^{14}C]-benzoate serves as a sensitive, simple, and efficacious probe for the production of an oxidative radical or radicals generated during microsomal electron transport. This oxidant appears to resemble ·OH in its oxidizing properties.

Earlier investigations have established that benzoic acid was decarboxylated upon interaction with hydroxyl radicals (·OH)[1] generated by radiolysis of aqueous solutions (Matthews & Sangster, 1965; Hoigne & Bader, 1975). More recently, Sagone et al. (1980) extended this concept by demonstrating that benzoate also was decarboxylated by ·OH that was generated during the coupled oxidation of xanthine by xanthine oxidase and by ·OH or a similar oxidant generated by granulocytes during the phagocytosis of zymosan particles. The production of $^{14}CO_2$ from [7-^{14}C]benzoate was used to investigate the mechanism of ·OH generation by phagocytic cells (Sagone et al., 1980).

The current report concerns the use of benzoate as a probe for the detection of oxy radicals generated by another biological system, NADPH-dependent, microsomal electron transfer. Microsomes produce $O_2^{-}\cdot$ and H_2O_2 during electron transport (Hildebrandt & Roots, 1975; Thurman et al., 1972; Aust et al., 1972; Prough & Masters, 1973; Nordblom & Coon, 1977; Dybing et al., 1976; Fong et al., 1973). Two possible loci of $O_2^{-}\cdot$ production are the autoxidation of oxycytochrome P-450 and the autoxidation of reduced NADPH–cytochrome P-450 reductase. H_2O_2 is produced from the spontaneous dismutation of $O_2^{-}\cdot$. H_2O_2 can serve as a precursor of ·OH via either a Fenton reaction (Walling, 1975) or an iron-catalyzed Haber–Weiss reaction (McCord & Day, 1978, Halliwell, 1978). During microsomal electron transport, ethylene was produced from methional or from KTBA, whereas methane gas was generated from Me_2SO (Cohen & Cederbaum, 1979, 1980). The oxidation of these ·OH scavengers to products known to arise from their interaction with ·OH suggested that microsomes generate an oxy radical with oxidizing properties similar to those of ·OH. However, ethylene production from methional or KTBA is not specific for the detection of ·OH (Pryor & Tang, 1978), and recent results indicate that formaldehyde, not methane, represents a major product of the interaction of Me_2SO with ·OH (Klein et al., 1981). In view of the fact that benzoate was shown to inhibit the oxidation of alcohols by the microsomes, a system dependent on the interaction of the alcohols with metabolically generated ·OH (Cederbaum et al., 1978, 1979, 1981), it appeared that microsomes might be capable of catalyzing the decarboxylation of benzoate. The data reported herein demonstrated that $^{14}CO_2$ production from [7-^{14}C]benzoic acid

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[1] Abbreviations: ·OH, hydroxyl radical or a species with the oxidizing power of the hydroxyl radical; Me_2SO, dimethyl sulfoxide; KTBA, 2-oxo-4-(methylthio)butyric acid; $O_2^{-}\cdot$, superoxide anion radical; EDTA, ethylenediaminetetraacetic acid.

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