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# Regulation of the activity of branched-chain 2-oxo acid dehydrogenase (BCODH) complex by binding BCODH kinase

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**Abstract Branched-chain 2-oxo acid dehydrogenase (BCODH) kinase is responsible for inactivation of BCODH complex by phosphorylation of the complex. Activity of the kinase towards its substrate, the E1 component of the BCODH complex, is known dependent upon binding of the kinase to the E2 component. The possible existence as well as importance of unbound mitochondrial BCODH kinase has been largely ignored in previous studies. Evidence is presented here for the existence of free and bound BCODH kinase in the matrix space of rat liver mitochondria. Furthermore, in female rats, in which diurnal variations in liver BCODH complex and kinase activities occur, the amount of the kinase bound to the complex changes between morning and evening without a change in total kinase protein. Activity of the kinase correlates with the amount of bound rather than total kinase protein, suggesting only the bound form is active. Changes in amount of kinase bound and therefore active appear responsible for diurnal variation in BCODH complex activity in the female rat. We propose that change in the amount of bound BCODH kinase is a key feature of a novel regulatory mechanism for determining the activity state of the BCODH complex.** 

*Key words:* Branched-chain 2-oxo acid dehydrogenase kinase; Branched-chain 2-oxo acid dehydrogenase complex; Free form; Bound form; Rat liver mitochondrion

## 1. Introduction

The mammalian branched-chain 2-oxo acid dehydrogenase (BCODH) complex, an intramitochondrial multienzyme complex (3-methyl-2-oxobutanoate dehydrogenase (lipoamide) (E1: EC 1.2.4.4), dihydrolipoamide acyltransferase (E2: no EC number), and dihydrolipoamide dehydrogenase (E3: EC 1.8.1.4)), catalyzes the oxidative decarboxylation of the transamination products of the branched-chain amino acids (valine, leucine and isoleucine) [1]. The BCODH complex is subject to covalent modification; BCODH kinase (EC 2.7.1.115) [2,3] inactivates the complex by phosphorylation of the E1 component of the complex and BCODH phosphatase (EC 3.1.3.52) [4,5] reactivates it by dephosphorylation.

The regulation of the activity of BCODH complex through kinase-mediated phosphorylation has been extensively studied in rat tissues [6–10]. An inverse correlation is found under various physiological conditions between the activity state (the percentage of active, non-phosphorylated form) of BCODH complex and the activity of BCODH kinase tightly associated with the complex. In many cases, a stable change in the kinase activity was accompanied by corresponding changes in level of the kinase protein bound to the complex [6–8] and kinase mRNA [6,7], suggesting BCODH kinase may be subject to transcriptional regulation in response to extracellular stimuli [11]. In studies in which kinase protein was quantitated [6–10], the bound or total form of the BCODH kinase was measured.

In the present study we examined the existence of the free (non-bound) form of BCODH kinase and its amount relative to bound form in rat liver mitochondria and found that the amount of the free form was greater than that of the bound form. Furthermore, we show here that the diurnal variation of the activity state of hepatic BCODH complex, which was exclusively observed in female rat, is associated with a marked change in the amount of bound kinase with no change in total kinase.

## 2. Materials and methods

### 2.1. Materials

Protein A-agarose was purchased from Upstate Biotechnology (Lake Placid, NY, USA). [$^{125}$I]anti-rabbit immunoglobulin was from Amersham Pharmacia Biotech (Tokyo, Japan). Broad specificity phosphoprotein phosphatase was prepared from bovine heart according to the method reported previously [12]. Antisera against each component (E1 and E2) of BCODH complex were prepared using purified E1 and E2 components from isolated rat liver BCODH complex and antiserum against BCODH kinase was prepared using recombinant BCODH kinase as reported previously [13]. Gonadectomized and sham-operated female rats were prepared as described previously [8]. All other reagents were of biochemical grade.

### 2.2. Animals

Female Sprague-Dawley rats aged 8 weeks (CLEA Japan, Tokyo, Japan) were used throughout the experiments. They were housed at 24°C in individual cages with light from 05:00 h to 17:00 h. They had free access to water and chow diet (CE2, CLEA Japan). All procedures involving animals were approved by the experimental animal care committee of Nagoya Institute of Technology.

### 2.3. Extraction of enzymes from rat livers

Livers were removed from anesthetized rats, immediately freeze clamped, and stored at −80°C until use. Frozen liver (50 mg) was homogenized in 5 volumes of ice-cold buffer A (10 mM Tris–HCl, pH

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7.5, 1 mM EDTA, 0.5% (w/v) Triton X-100, 2% (v/v) bovine serum, 10 μM *p*-toluenesulfonyl-L-phenylalanine chloromethyl ketone (TPCK), 10 μg/ml trypsin inhibitor, 1 μM leupeptin, and 5 mM dithiothreitol (DTT)). DTT was added as a solid to the buffer just prior to use throughout the procedures. The homogenate was centrifuged at 10 000×*g* for 30 min at 4°C to provide supernatant designated as crude liver extract. The protein concentration in the extract was determined using the Bio-Rad protein assay dye reagent.

*2.4. Immunoprecipitation*

Before immunoprecipitation, the liver extracts (200 μl containing 8 mg of protein) were precleared by incubation with protein A-agarose (10 μl) overnight at 4°C and centrifugation to limit non-specific precipitation. The liver extracts obtained were then subjected to immunoprecipitation with immunoadsorbents of protein A-agarose associated with polyclonal antibodies against BCODH E1 (α+β subunits) or BCODH kinase. The immunoadsorbents used were prepared by incubating 5 μl of the antiserum with protein A-agarose (10 μl) suspended in 0.5 ml of buffer A for 2 h at 4°C. The cleared extracts were then incubated with the appropriate immunoadsorbent for 5 h at 4°C. After centrifugation, the supernatant was kept for immunoblot analysis, and the protein A-agarose pellet was washed five times with 500 μl of buffer A and eluted by boiling in equal volume of gel electrophoresis sample buffer (125 mM Tris–HCl, pH 6.8, 10% (v/v) 2-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol and 0.01% (w/v) bromo phenol blue).

*2.5. Immunoblotting and immunodetection*

For immunoblotting analysis, proteins in the liver extracts (50 μg of protein) or immunoprecipitates were separated by SDS–PAGE according to the method of Laemmli [14] and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) by the semidry method. Non-specific binding to the membrane was blocked with 3% (w/v) bovine serum albumin in TBST (20 mM Tris–HCl, pH 7.5, 0.5 M NaCl and 0.05% (v/v) Tween-20). The blots were incubated with the primary antibody in blocking buffer, washed three times with TBST and further incubated with the secondary antibody, [$^{125}$I]anti-rabbit immunoglobulin, in blocking buffer. The radioactivities associated with protein bands on the membrane were analyzed by a laser image analyzer (Fuji BAS1000, Fuji Film, Tokyo, Japan).

For stripping, the membrane was incubated in 62.5 mM Tris–HCl, pH 6.7, 100 mM 2-mercaptoethanol and 2% (w/v) SDS for 30 min at 50°C, and then extensively washed with TBST. The blot was blocked and reprobed as described above. Equivalent protein loading for immunoprecipitated proteins was verified by reprobing with antiserum raised against the BCODH E1 component.

*2.6. Rat liver mitochondria preparation*

Rat liver mitochondria were prepared essentially as described previously [15,16]. All steps were carried out at 0–4°C. Livers were rapidly removed from anesthetized rats, rinsed in ice-cold 0.25 M sucrose and homogenized in 4 volumes of ice-cold buffer B (0.25 M sucrose, 20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 10 μM TPCK, 10 μg/ml trypsin inhibitor, 1 μM leupeptin and 5 mM DTT). After centrifugation at 700×*g* for 10 min, the supernatant was removed and subjected to centrifugation at 10 000×*g* for 10 min. The resultant postmitochondrial supernatant was retained. The mitochondrial pellet was resuspended in buffer B and re-centrifuged at 10 000×*g*, and this washing procedure was further repeated three times. Following the final wash, mitochondria were suspended in buffer B. The protein concentrations and the activities of citrate synthase as a mitochondrial marker enzyme in the liver extract, mitochondrial extract, and postmitochondrial supernatant were determined. The release of mitochondrial enzymes into the postmitochondrial supernatant was assessed by the activity of citrate synthase. The mitochondrial extract prepared using buffer A was also subjected to immunoprecipitation as described above.

*2.7. Enzyme assays*

Actual and total activities of BCODH complex were determined spectrophotometrically by measuring the rate of NADH production [17]. Dephosphorylation of the complex for measuring the total activity was performed using broad specificity phosphoprotein phosphatase [12]. One unit of the complex catalyzed the formation of 1 μmol of NADH/min. The activity state was calculated as the ratio of actual activity (activity of the active form of the enzyme in vivo) to total activity. The assay of BCODH kinase was performed by measuring ATP-dependent inactivation of BCODH as described previously [8]. Kinase activity is expressed as the first order rate constant of BCODH inactivation. Citrate synthase activity was determined as described by Shepherd and Garland [18].

*2.8. Statistical analysis*

Results are expressed as means ± S.E.M. Statistical analysis was performed using the Student's *t*-test for unpaired samples.

## 3. Results and discussion

### *3.1. Immunochemical analyses of total, bound, and free (non-bound) forms of BCODH kinase*

Evidence has been provided in previous studies that BCODH kinase binds tightly to the BCODH complex and that binding is required for phosphorylation and therefore regulation of BCODH complex activity [6–10]. However, little or no attention has been paid to BCODH kinase not associated with the complex that might exist in mitochondria. In the present study we examined whether all BCODH kinase expressed in rat liver is tightly associated with the BCODH complex. Since we previously reported that livers obtained from female rats in the evening of a day contain fairly low activity state of BCODH complex and high kinase activity

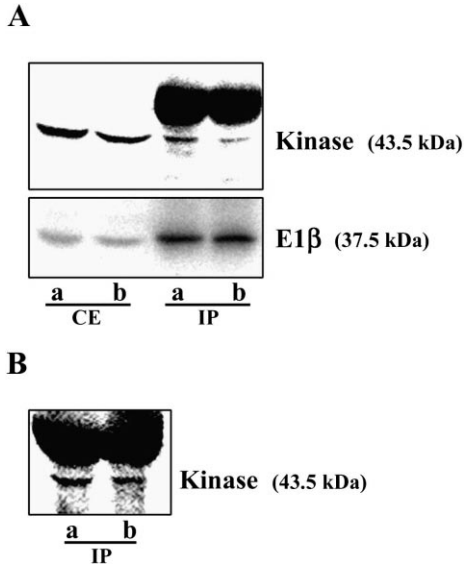


Fig. 1. Analysis of BCODH kinase protein levels in rat liver crude extracts. A: Immunoblot analysis of BCODH kinase protein in crude extracts of rat livers with different activity states of BCODH complex. The activity state of BCODH complex was 4% for sample *a* and 71% for sample *b*, although the total activity of the BCODH complex was very similar (approximately 1400 mU/g wet wt.) in both samples. Equal amounts of liver crude extract (CE) were subjected to SDS–PAGE and blotted onto PVDF membrane. The crude extracts were immunoprecipitated (IP) with antiserum raised against BCODH E1 (α+β subunits) and subjected to SDS–PAGE and blotting. Immunoblotting was performed with antiserum raised against BCODH kinase (upper panel). The large bands above the kinase in IP lanes are due to IgG. The blots were stripped and the membrane reprobed with antiserum raised against BCODH E1 (α+β subunits) to verify the same amount of loading of BCODH protein between samples (lower panel). B: Immunoblot analysis of total BCODH kinase protein present in crude liver extracts. The crude extracts from samples *a* and *b* were immunoprecipitated (IP) with antiserum raised against BCODH kinase and analyzed by immunoblotting with the same antiserum.

and that gonadectomy renders the reverse situation [8], we used in this study livers from sham-operated (sample *a*) and gonadectomized (sample *b*) female rats: the BCODH complex was 4 and 71% active, respectively, and kinase activities were 0.82 and 0.27 $min^{-1}$, respectively (Fig. 1A). Crude liver extracts from these liver samples were analyzed by immunoblotting. The BCODH complex present in these crude extracts was first isolated by immunoprecipitation with antiserum raised against the E1 component of the BCODH complex. The amount of kinase associated with the complex was determined by immunoblot analysis with antiserum raised against BCODH kinase. This procedure allows determination of the amount of kinase bound to the BCODH complex relative to a subunit of the complex as reported previously [6]. The amount of samples loaded to SDS–PAGE and blotted was normalized on the basis of the amount of the E1β subunit of the complex. As shown in Fig. 1A (IP), the level of kinase protein bound to the complex was greater in sample *a* than in sample *b*, with the relative amounts correlating directly with BCODH kinase activity and inversely with BCODH complex activity state. These findings are in agreement with results published previously [6–8].

In contrast to the above results, no difference in the amounts of BCODH kinase protein was observed by immunoblot analysis of total crude liver extract protein. This held true regardless of whether data were expressed on the basis of total loaded protein or the relative amount of BCODH E1β protein (Fig. 1A, CE). Furthermore, the amount of BCODH kinase present in crude liver extracts was found to be considerably greater than the amount determined to be tightly bound to the complex by immunoprecipitation with E1 antiserum. This finding was confirmed with an independent experiment in which antiserum against BCODH kinase was used to immunoprecipitate BCODH kinase from the crude liver extracts. Immunoblot analysis of the resulting immunoprecipitates again revealed similar amounts of kinase protein in both samples (Fig. 1B). These results provide evidence for the presence of free BCODH kinase in crude liver extracts and further suggest that a fairly large proportion of the kinase present in liver exists in the free form.

Because activity of the BCODH kinase is quite low in crude tissue extracts, it has become routine to precipitate the BCODH complex from such extracts with polyethylene glycol prior to assay [17]. Not previously investigated is whether polyethylene glycol precipitates both the free and the bound forms of BCODH kinase. This was investigated in this study with the finding that both forms of the kinase are precipitated by polyethylene glycol at the concentration (9%) normally used for quantitative precipitation of the complex (data not shown). In spite of precipitating both forms, the activity of the kinase measurable in polyethylene glycol precipitates correlates with the amount of bound kinase protein rather than the total kinase protein, suggesting the free form of the kinase does not have the capability for binding and phosphorylation of the complex.

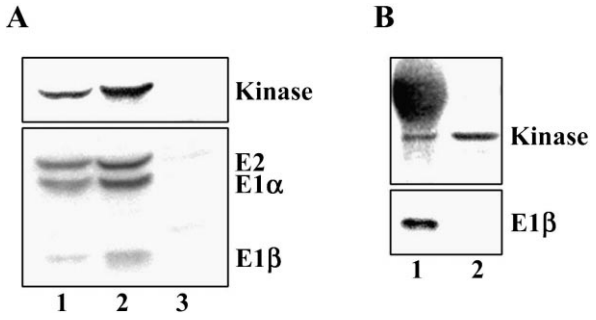


Fig. 2. Immunodetection of bound and free forms of BCODH kinase in rat liver mitochondria. A: Localization of BCODH kinase protein. The proteins (50 μg) present in the crude extract (lane 1), mitochondrial extract (lane 2) and postmitochondrial supernatant (lane 3) prepared from rat liver were resolved by SDS–PAGE. Proteins were blotted onto PVDF membranes and probed with antiserum raised against BCODH kinase (upper panel). The blots were stripped and the membrane was reprobed with antiserum raised against BCODH E1 (α+β subunits) +E2 (lower panel). B: Immunoblot analysis of BCODH kinase protein in the mitochondrial extract. Mitochondrial extract (shown at lane 2 in A) was immunoprecipitated with antiserum raised against BCODH E1 (α+β subunits) (lane 1). Protein in the supernatant resulting from the immunoprecipitation was also analyzed (lane 2). Immunoblotting was performed with kinase antiserum (upper panel), and the blots were stripped and the membrane was reprobed with BCODH E1 (α+β subunits) antiserum (lower panel).

Because the above results could be explained by the presence of free BCODH kinase in the cytoplasm, we determined the subcellular distribution of the BCODH kinase. The same approach to identify the kinase protein was applied to mitochondrial and postmitochondrial fractions prepared from rat liver. Based on activity of the mitochondrial marker enzyme citrate synthase, the postmitochondrial fraction prepared for this study was nearly void of contamination with mitochondrial matrix proteins. As would be expected for a protein located exclusively in the mitochondria, kinase protein was detectable by immunoblot analysis of crude liver extract and mitochondrial extract, but not in the postmitochondrial fraction (Fig. 2A). This finding indicates that free and bound forms of the kinase exist in mitochondria. To confirm existence of the free form of the kinase, the mitochondrial extract was subjected to immunoprecipitation with antiserum raised against BCODH E1. As shown in Fig. 2B, the kinase was contained in both immunoprecipitate and supernatant fractions, but the BCODH complex was contained only in the precipitate, clearly indicating the existence of the free form of the kinase.

Table 1
Diurnal changes in the levels of hepatic BCODH complex and its kinase in female rats

| Time point | BCODH complex activity (mU/g wet wt.) | | Activity state (%) | BCODH kinase ($min^{-1}$) |
|---|---|---|---|---|
| | Actual | Total | | |
| Morning (09:00 h) | 883 ± 74 | 1088 ± 42 | 81 ± 4 | 0.39 ± 0.08 |
| Evening (17:00 h) | 114 ± 58[a] | 1192 ± 118 | 9 ± 4[a] | 0.85 ± 0.14[a] |

Rats were sacrificed either at the morning (09:00 h) or evening (17:00 h). Liver extracts were prepared and the activities were determined as described in Section 2. Results are means ± S.E.M. for three rats per time point.
[a]Significantly different from the results at other time point ($P < 0.05$). BCODH kinase activity is expressed as a first order rate constant of BCODH inactivation.

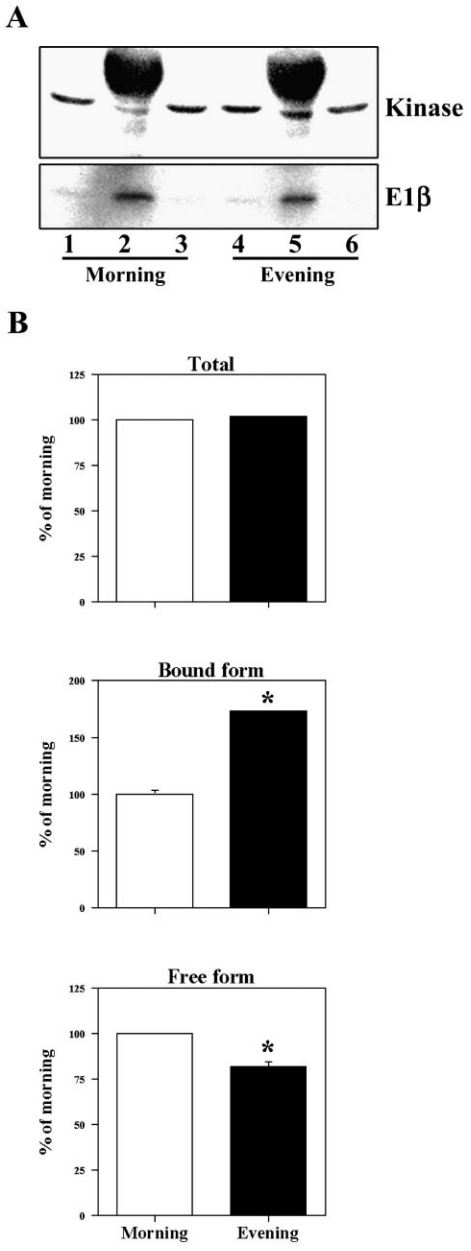


Fig. 3. Diurnal changes in bound and free forms of BCODH kinase in liver mitochondria of female rats. A: Immunoblot analysis of BCODH kinase protein in mitochondrial extracts at two different time points (typical data). Rats were sacrificed either in the morning (09:00 h) or evening (17:00 h) in a day. Mitochondrial extracts were prepared from rat livers and subjected to SDS–PAGE (lanes 1 and 4). Mitochondrial extracts were immunoprecipitated with BCODH E1 (α+β subunits) antiserum (lanes 2 and 5) and the supernatants after immunoprecipitation were also analyzed (lanes 3 and 6). Immunoblotting was performed with kinase antiserum (upper panel), and the blots were stripped and the membrane was reprobed with BCODH E1 (α+β subunits) antiserum (lower panel). B: Quantification of BCODH kinase protein. The quantification of the signal intensities was carried out using a laser image analyzer (Fuji BAS1000). Results are means ± S.E.M. for three rats in each time point. The data of the evening group are expressed as percentages of the morning group. *Significantly different from the morning ($P < 0.05$).

### 3.2. Diurnal change in the BCODH complex activity associated with alteration in the ratio of bound to free form of BCODH kinase

It has been reported that female rats exhibit a clear diurnal variation in the activity state of BCODH complex: high activity of the complex in the morning (absorptive period) and low activity in the evening (post-absorptive period) [8]. In the present study, we investigated the relationship between the activity state of BCODH complex and the ratio of bound to free form of the kinase in female rat liver. Three female rats were sacrificed in the morning (09:00 h) and in the evening (17:00 h) of the same day. Regardless of the time when the animals were killed, the total activity of BCODH complex was almost equal (Table 1). However, the activity state of the complex was 9-fold higher in the liver of animals killed in the morning versus animals killed in the evening (Table 1). As expected, the activity of BCODH kinase was ~2-fold higher in the evening than in the morning (Table 1). Thus an inverse correlation between the activity state of BCODH complex and the kinase activity was observed as reported previously [8].

The total amount of the kinase protein determined by immunoblot analysis of mitochondria isolated from the livers of rats killed at the two time points was nearly identical (Fig. 3). However, the bound form of the kinase was 1.7-fold greater in the evening than in the morning (Fig. 3), in accordance with the results of the kinase activity. On the other hand, the amount of free form of the kinase protein was only 20% less in the evening than in the morning (Fig. 3). These results indicate that most of the kinase exists in liver mitochondria in the free form and suggest that induced alteration in the ratio of bound to free form of the kinase plays an important role in regulation of the activity state of BCODH complex in rat liver.

### 3.3. Conclusion

By immunoprecipitation techniques we have demonstrated the existence of the free (non-bound) form of BCODH kinase in rat liver mitochondria. Our results suggest that the majority of the kinase is in a free form. Only the small amount of the kinase that is bound contributes to regulation of BCODH complex activity. The mechanism that induces a change in BCODH kinase binding to the BCODH complex remains to be defined.

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