Am J Hum Genet 36:802–807, 1984

Direct Physical Evidence for Stabilization of Branched-Chain α-Ketoacid Dehydrogenase by Thiamin Pyrophosphate

S. C. HEFFELFINGER,[1] E. T. SEWELL,[2] L. J. ELSAS,[3] AND D. J. DANNER[3]

SUMMARY

Branched-chain α-ketoacid dehydrogenase is a multienzyme complex composed of four subunits. The 46,500-dalton protein is a subunit of the decarboxylase component, which is selectively digested by chymotrypsin. Two peptides of apparent mol. wts. of 36,000 and 15,000 result with loss of enzyme activity. When the complex is saturated with thiamin pyrophosphate and ketoacid substrate, digestion by chymotrypsin does not occur. These data provide direct physical evidence for the stabilization of the complex by the presence of the vitamin B1-derived cofactor.

INTRODUCTION

Branched-chain α-ketoacid dehydrogenase (BCKD) is a multienzyme complex associated with the inner membrane of mitochondria and functions as the committed reaction for catabolism of the branched-chain amino acids [1–3]. Inherited mutations in man, known as maple syrup urine disease (MSUD), specifically affect the function of this complex [4]. Four peptides comprise the three enzymes of this complex, and deleted or altered structural genes for any of the peptides could cause a malfunction resulting in the disease state [5].

Since the BCKD complex requires a vitamin-derived cofactor, thiamin pyrophosphate (TPP), which combines with the decarboxylase component of BCKD to form the α-ketoacid binding site, a vitamin-responsive form of MSUD was hypothesized [6]. Clinical trials in selected patients showed that BCKD complex activity was improved when the protein-restricted diet was supplemented with thiamin [7–12]. Thiamin given at > 100 mg/day led to decreased urinary output

Received October 31, 1983; revised January 19, 1984.

This study was supported by grant HD08388 from the U.S. Public Health Service and from the Emory University Research Committee.

[1] Department of Pathology, Baylor Medical School, Houston, TX 77030.

[2] Center for Disease Control, Atlanta, GA 30333.

[3] Division of Medical Genetics, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30322.

of branched-chain ketoacids and increased BCKD activity in peripheral leukocytes. In other studies, hepatic BCKD also was stimulated by supplementation of normal diet with 100 mg/day of thiamin in individuals with normal BCKD activity [13]. When purified bovine liver BCKD complex was saturated with TPP, the complex was protected against inactivation by heat and chymotrypsin digestion and underwent a conformational change upon the binding of this cofactor [14]. It was postulated that a conformational change led to the stabilization of the BCKD complex, which would explain the in vivo responses to thiamin and TPP [8, 9]. Here, we provide direct physical proof of protection against proteolytic digestion of a peptide within the BCKD complex in the presence of TPP.

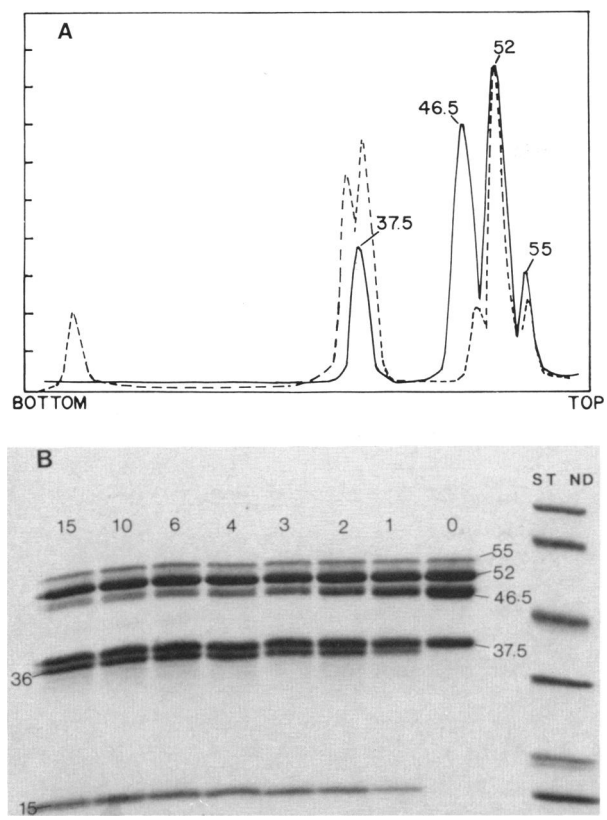

FIG. 1.—*A*, Densitometry tracing of proteins comprising the BCKD complex. *Solid line* represents the protein composition of the complex depleted of TPP and prior to chymotrypsin digestion, *lane 0* of *B*. *Dashed line* presents the proteins of the BCKD complex after 15 min of chymotrypsin digestion, *lane 15* of *B*. *B*, Resolution of BCKD proteins by gel electrophoresis as described in MATERIALS AND METHODS. BCKD was depleted of TPP as described in the text and digested with chymotrypsin (0.5 μg/0.23 mg BCKD protein). Ten-μl aliquots were taken for electrophoresis at 0–4, 6, 10, and 15 min as indicated *from right to left across the top of each gel. Nos. on the sides of the gel* are molecular weights of proteins × 10^3 as calculated from a curve generated with molecular weight markers (STAND).

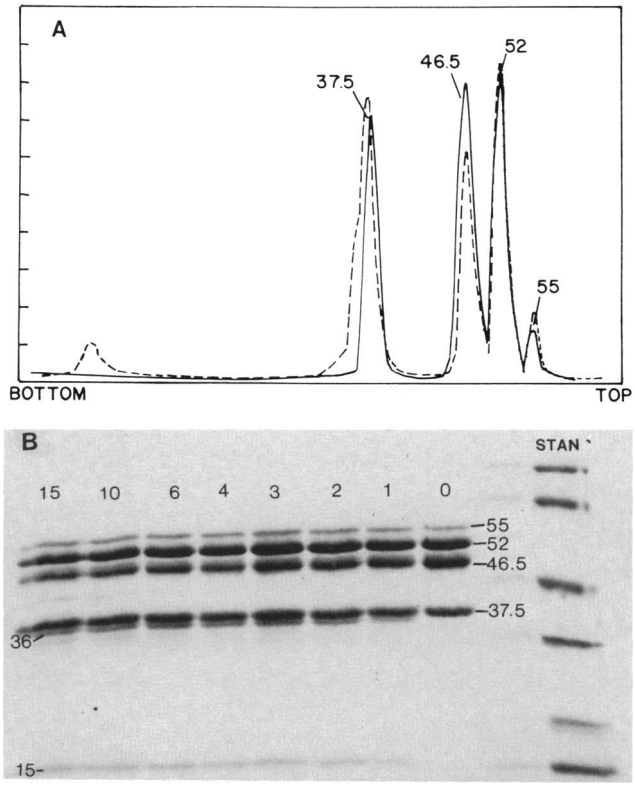

FIG. 2.—*A*, Densitometry tracing of proteins comprising the BCKD complex before (*solid line*) and after (*dashed line*) 15 min of digestion with chymotrypsin in the presence of 0.2 mM TPP. *B*, Proteins of the BCKD complex resolved by SDS-PAGE during the digestion by chymotrypsin in the presence of 0.2 mM TPP. Details as described in figure 1*B*.

MATERIALS AND METHODS

All reagents were made in deionized water with reagent-grade or ultra-pure chemicals. TPP and chymotrypsin were purchased from Sigma, St. Louis, Mo. Reagents for electrophoresis were obtained from Bio-Rad, Richmond, Calif.

BCKD was prepared from bovine liver mitochondria as described [5]. Subunits of the BCKD complex were resolved by electrophoresis in a 10%–16% gradient polyacrylamide gel in the presence of sodium dodecyl sulfate and 5% β-mercaptoethanol using the conditions of Laemmli [15]. Details of the conditions for protease digestion are in the legends of figures 1–3.

Densitometry was done by scanning photographic negatives of the gel electrophoresis resolved proteins of the BCKD complex with an Helena Cliniscan Densitometer.

RESULTS

When BCKD was dialyzed against 30 mM potassium phosphate, pH 7.2, containing 0.1 mM EDTA, for 11 hrs, endogenous TPP was removed so that activity depended upon addition of this cofactor [5]. Incubation of the TPP-depleted BCKD complex with chymotrypsin led to a selective digestion of the 46,500-dalton subunit into two fragments of 36,000 and 15,000 daltons. Figure 1*A* com-

pares gel traces of the proteins in the BCKD complex resolved by electrophoresis (fig. 1*B*) after 0 and 15 min of chymotrypsin digestion of the TPP-depleted complex. The time course of this degradation paralleled the time-dependent loss of catalytic activity [14]. Addition of 0.2 mM TPP to the BCKD complex prior to incubation with chymotrypsin retarded the selective digestion as seen by the decreased production of the 36,000- and 15,000-dalton peptides during the 15-min digestion (fig. 2*A* and *B*). When both α-ketoacid and TPP were present, no chymotrypsin digestion occurred (fig. 3*A* and *B*). Although the same amount of protein was loaded on each gel for the experiments described in figures 1–3, staining was minimal in figure 3. We have attempted to compensate for this difference in the densitometry tracing of the gel where peak heights for the 52,000-dalton protein were adjusted to approximately equal density. This effect of TPP was not due to inactivation to chymotrypsin since the artificial substrate, succinyl-L-phenylalanine-*p*-nitroanilide was cleaved both in the absence and presence of TPP. (Relative rate without TPP, 0.011 ΔA/min, and with TPP, 0.016

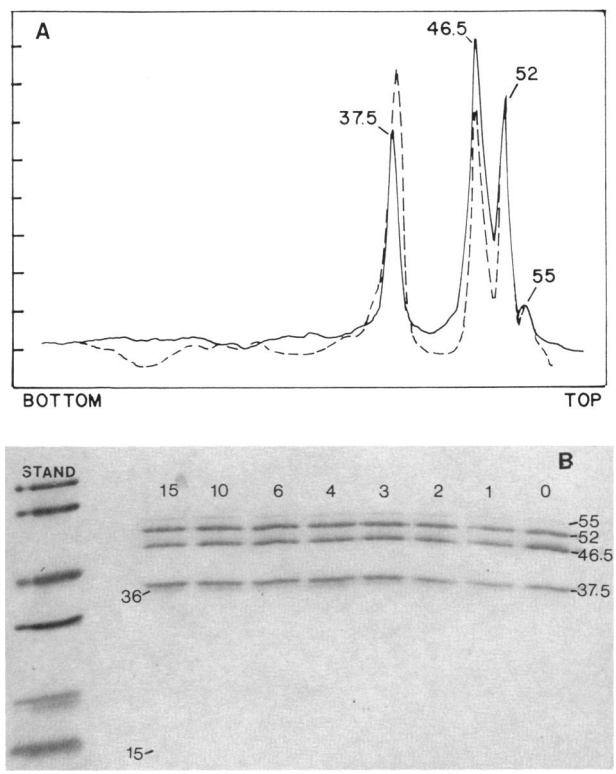

FIG. 3.—*A*, Densitometry tracing of proteins comprising the BCKD complex before (*solid line*) and after (*dashed line*) 15 min of digestion with chymotrypsin in the presence of 0.2 mM TPP and 0.2 mM α-ketoisocaproate. *B*, Proteins of the BCKD complex resolved by SDS-PAGE during the digestion by chymotrypsin in the presence of 0.2 mM TPP and 0.2 mM α-ketoisocaproate. Details are described in figure 1*B*.

ΔA/min.) Of the four proteins forming the BCKD complex, the 46,500-dalton component represents about 35%. In the absence of TPP, this percentage is reduced to 10 by 5 min while the new peptides of 36,000 and 15,000 daltons rise to 23% and 4%, respectively. More than 30 min of exposure to chymotrypsin in the absence of TPP was required to see a general digestion of all the proteins.

DISCUSSION

Stabilization of enzymes against proteolytic degradation by the presence of cofactors has been known for more than 20 years [16, 17]. The use of vitamin supplementation of diets for individuals with inherited defects of specific enzyme functions was tried on this basis [7]. Stabilization of mutant enzyme by its vitamin-derived cofactor has been demonstrated to support this rationale [8, 9, 18, 19]. Our data with chymotrypsin digestion of BCKD demonstrates a physical basis for enzyme stabilization by the cofactor.

It has been suggested that chymotrypsin mimics the action of some mitochondrial proteases that are responsible for turnover of the mitochondrial proteins [14]. This protection against the action of chymotrypsin by TPP supports our earlier hypothesis of decreased turnover of BCKD in the presence of this cofactor and suggests a role for TPP in addition to its function to form the catalytic site on the decarboxylase subunit of BCKD. These results provide evidence that the 46,500-dalton protein is the TPP-binding site for the decarboxylase function. This is further supported by the finding that when the branched-chain ketoacid substrate is present along with TPP, full protection against digestion resulted (fig. 3). Although the 37,500-dalton component is reported to be the β-subunit of the decarboxylase [20], no digestion of this subunit occurs under these conditions. The data also offer a physical explanation for one form of vitamin-responsive MSUD.

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