Volume 288, number 1,2, 95–97 FEBS 10052 August 1991

ADONIS 001457939100760P

Immunochemical identification of branched-chain 2-oxo acid dehydrogenase kinase

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Received 24 May 1991

Branched-chain 2-oxo acid dehydrogenase kinase was characterized using anti-kinase polyclonal antibodies. The antibodies were purified from rabbit antiserum by an epitope selection method. The antibodies bound only to a 44 kDa polypeptide in the dehydrogenase–kinase complex and inhibited the kinase activity, substantiating that the 44 kDa polypeptide is the catalytic subunit of the kinase. The purified liver dehydrogenase–kinase complex, but not either the purified heart complex or the partially purified liver complex, contained 2 additional polypeptides of lower molecular weight which also reacted with the anti-kinase antibodies, suggesting that the liver kinase is subject to proteolytic degradation during purification.

Branched-chain 2-oxo acid dehydrogenase kinase; Branched-chain 2-oxo acid dehydrogenase complex; Anti-kinase antibody; Low-protein fed rat; Rat liver; Rat heart

1. INTRODUCTION

Branched-chain 2-oxo acid dehydrogenase (BCODH) complex (EC 1.2.4.4. + dihydrolipoamide acyltransferase (no EC number) + EC 1.8.1.4) is the rate-limiting enzyme in the catabolism of branched-chain amino acids (valine, leucine and isoleucine) [1]. This enzyme complex is subject to regulation by covalent modification. BCODH kinase (no EC number) is responsible for phosphorylation and inactivation of the E1α component of the complex [2–5]. Recently, we have purified BCODH kinase from rat liver and rat heart and partially characterized the kinase [6]. In this paper, we report further characterization of the kinase using monospecific anti-kinase polyclonal antibodies.

2. MATERIALS AND METHODS

2.1. *Materials*

Rat heart and liver BCODH-kinase complex, BCODH kinase and kinase-depleted BCODH were prepared as described in [6]. Anti-kinase antibodies were prepared by immunization of a rabbit with the purified heart kinase preparation as described in [7] except that the 4th injection of the antigen was omitted, and the antibodies were purified from the antiserum by the epitope selection method of Kelly et al. [8]. For the study of the kinase in rapidly purified liver BCODH-kinase complex, 4 male Sprague-Dawley rats (CLEA Japan, Tokyo) with initial body weights of 150–164 g were fed ad libitum a semi-synthetic diet containing 5% casein for 2 weeks. The animals were immobilized by cervical dislocation and livers removed and freeze-clamped (< 10 s) at the temperature of liquid nitrogen. Other materials used were described in [6].

2.2. *Methods*

BCODH complex was partially purified from livers of rats fed the diet described above for Western blot analyses; BCODH complex was extracted from livers as described in [9] and was purified by a one cycle of Phenyl-Sepharose column chromatography (using a 2 ml column) and polyethylene glycol precipitation as described in [10]. SDS-PAGE was performed by the method of Laemmli [11]. Samples for electrophoresis were treated as described in [10]. Electroblotting of SDS-PAGE gels and immunostaining of blotted filters were carried out as described in [12]. BCODH activity was spectrophotometrically measured as described in [10]. Protein determination was by the BCA method using bovine serum albumin as a standard [13].

3. RESULTS AND DISCUSSION

3.1. *Specificity of the purified anti-kinase antibodies*

The specificity of the purified anti-kinase antibodies was examined by Western blot analysis. The purified antibodies bound only to the 44 kDa polypeptide in the heart BCODH-kinase complex preparation (Fig. 1B, lane 2). Kinase-depleted BCODH complex from the liver BCODH-kinase complex preparation showed only a faint band at 44 kDa in the analysis (Fig. 1B, lane 4). On the other hand, the purified liver BCODH-kinase complex preparation contained 3 polypeptides which reacted with the purified antibodies; one was the 44 kDa polypeptide and the others were polypeptides with

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Abbreviations: BCODH, branched-chain 2-oxo acid dehydrogenase; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

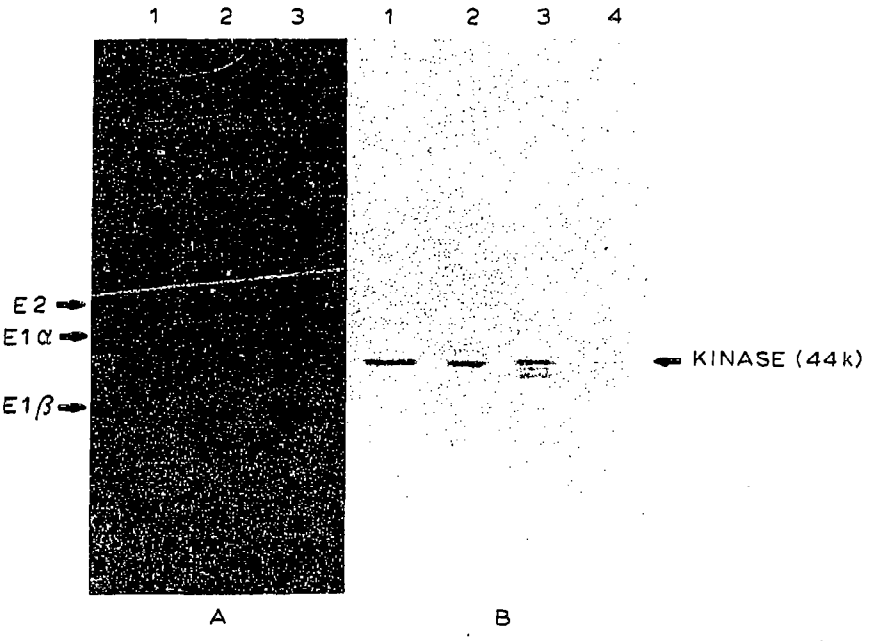

Fig. 1. Specificity of purified anti-kinase antibodies examined by Western blot analysis. Lane 1, almost purified heart kinase (0.6 μg); lane 2, purified heart BCODH-kinase complex (2 μg); lane 3, purified liver BCODH-kinase complex (2 μg); lane 4, liver kinase-depleted BCODH complex (2 μg). Proteins were resolved by SDS-PAGE. The proteins on blotted filters were detected by Coomassie blue staining (panel A) or by immunostaining with the purified anti-kinase antibodies (panel B). The 44 kDa polypeptide was considered to be BCODH kinase in the previous paper [6].

slightly lower molecular weights than 44 kDa (Fig. 1B, lane 3). Two independently purified preparations of the liver BCODH-kinase complex contained the same three immunoreactive protein bands. To examine whether these three polypeptides actually exist in intact rat liver or correspond to an artifact of liver BCODH-kinase complex purification, partially purified BCODH complex preparations were obtained from freeze-clamped rat livers. In our routine purification procedure for the BCODH-kinase complex [6], the livers are not freeze-clamped at the temperature of liquid nitrogen and, further, the liver extracts are incubated at 30°C for 50 min to fully activate the BCODH complex. Since it is possible that kinase protein could be degraded proteolytically, even when incubations were conducted in the presence of protease inhibitors, an experiment was carried out in which the livers were freeze-clamped and the incubation step was omitted during partial purification of the BCODH-kinase complex. As shown in Fig. 2, such preparations from rat livers contained only one band (44 kDa) which reacted with the purified antibodies, suggesting that the two polypeptides of lower molecular weight (Fig. 1B, lane 3) are proteolytic degradation products of the kinase produced during complete purification of the complex. Thus, it is reasonable to conclude that the purified antibodies generated for this study were specific for the 44 kDa polypeptide in the BCODH-kinase complexes of both rat liver and heart.

3.2. *Effect of the specific antibodies on kinase activity*

The effect of the purified antibodies on the BCODH

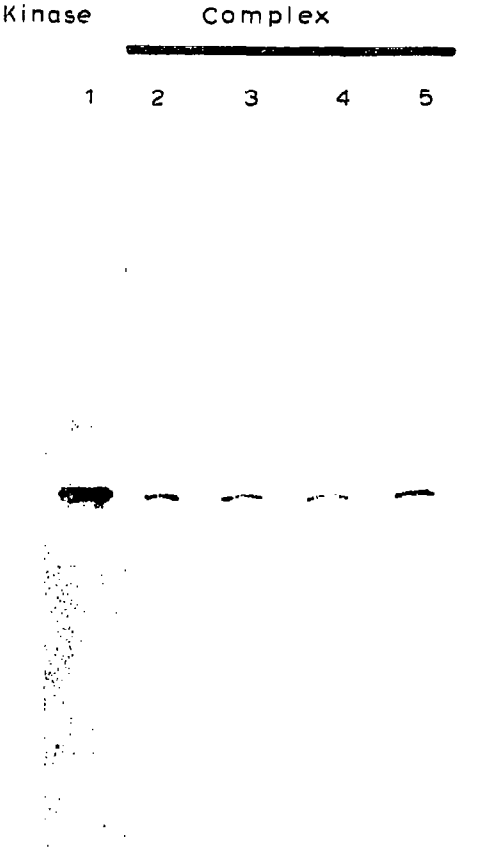

Fig. 2. Western blot analysis of partially purified BCODH complex from rat livers. 0.4 μg of the purified heart kinase (lane 1) and 50 μg of the partially purified BCODH complex from 4 rat livers (lanes 2-5) were subjected to SDS-PAGE.

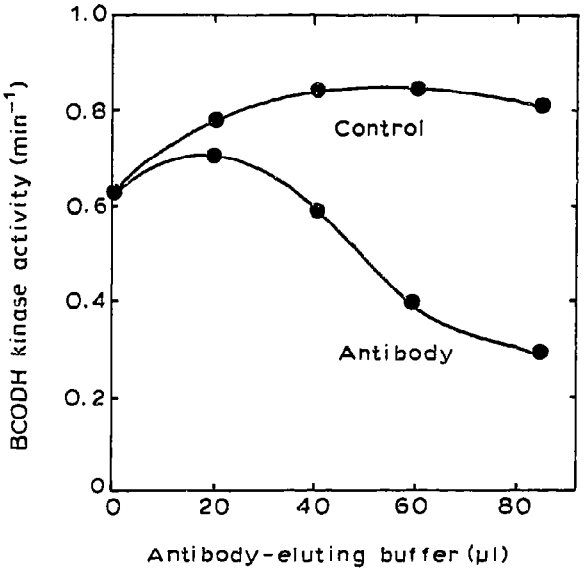

Fig. 3. Effect of the purified anti-kinase antibodies on the BCODH kinase activity. Purified liver BCODH-kinase (0.7 μg in 10 μl) was mixed with different amounts of the purified antibodies in the antibody-eluting buffer (indicated volumes) or with the eluting buffer without antibodies as a control. The mixtures were flushed with N_2 gas and then kept at 4°C for 24 h. The mixtures were finally made to 200 μl by addition of 22.5 μg of kinase-depleted BCODH complex and buffer consisting of 20 mM HEPES, 1.5 mM $MgCl_2$, 50 mM K_2HPO_4 and 2 mM dithiothreitol (pH 7.35 with NaOH). After pre-incubation at 30°C for 2 min, ATP (0.5 mM) was added and samples (20 μl) were taken at 0.5, 1 and 1.5 min directly into an assay mixture for BCODH activity. The kinase activity was expressed as a first order rate constant of BCODH inactivation.

kinase activity was examined in a reconstituted system consisting of the purified kinase plus kinase-depleted BCODH. Kinase activity was inhibited in an antibody dose-dependent manner, with approximately 65% inhibition being obtained as the maximal inhibition in this experiment (Fig. 3). In order to examine whether this amount of antibodies was sufficient to bind to all of the kinase protein under conditions of maximum inhibition, a mixture of the antibodies and kinase at the same ratio as that giving the maximum inhibition was subjected to immunoadsorption chromatography on a protein A-Sepharose column. Less than 4% of the kinase passed through the column, indicating that the antibodies bound to almost all the kinase protein and thereby substantiating that the 44 kDa polypeptide corresponds to the catalytic subunit of BCODH kinase.

Acknowledgements: This work was supported by grants from the U.S. Public Health Services (NIH DK 19259) and the Grace M. Showalter Residuary Trust to R.A.H., and the University of Tsukuba Project Research Fund to Y.S.

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