Antibodies to bovine liver branched-chain 2-oxo acid dehydrogenase cross-react with this enzyme complex from other tissues and species

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Antibodies were produced in rabbits against bovine liver branched-chain 2-oxo acid dehydrogenase (BCOAD) for three reasons. First, since activity of BCOAD varies among tissues within a species, it is necessary to ascertain whether isoenzymes exist within the different tissues. Second, it is of great interest to determine the structural similarity of BCOAD among mammalian species. Third, heritable defects of this multienzyme complex are known in humans, and techniques for studying BCOAD subunit composition in cells expressing the defect include the use of specific antibodies. Antibodies produced against BCOAD inhibit complex activity and cross-react with BCOAD from various tissues and species. By using rocket immunoelectrophoresis, the complex can be quantified in mitochondrial extracts. Each subunit is immunogenic, making the detection of subunit variants possible by the combined techniques of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and Western blotting. Finally, antibodies produced against pig heart lipoamide dehydrogenase cross-react with BCOAD, pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes, giving further evidence for the commonality of this subunit among these three mitochondrial complexes.

Branched-chain 2-oxo acid dehydrogenase (BCOAD; EC 1.2.4.4) is a multienzyme complex associated with the mitochondrial inner membrane of most mammalian tissues. The complex catalyses the oxidative decarboxylation of branched-chain 2-oxo acids (BCOA) via three enzyme components: a decarboxylase (E1), which releases CO₂, a core transacylase (E2), which transfers an acyl group to CoA, and lipoamide dehydrogenase (E3), which reduces NAD⁺ to NADH + H⁺:

This reaction is similar to that catalysed by the pyruvate dehydrogenase (EC 1.2.4.1) and 2-oxoglutarate dehydrogenase (EC 1.2.4.2) complexes. All three complexes are thought to be functionally similar and contain identical E3 subunits, although the E1 and E2 subunits are unique for each complex (Danner *et al.*, 1979; Pettit *et al.*, 1978).

The presence of BCOAD isoenzymes within a species has been in question. Tissue-perfusion studies with various amino acids in the perfusate and

whole-animal studies utilizing hormone and diet manipulation suggest that BCOAD activity may vary from tissue to tissue within a species (Odessey & Goldberg, 1972; Dohm *et al.*, 1976; Barakat *et al.*, 1977; Paul & Adibi, 1978, 1980; Buse, 1981). However, the inherited human disorder, branched-chain 2-oxoacidaemia, affects the function of BCOAD in all tissues of homozygous affected individuals (Dancis *et al.*, 1960, 1963). Specific

kinases and phosphatases which respectively inactivate or activate BCOAD have been reported. This has been offered as an explanation for the variation in BCOAD activity expressed in different tissues, but the question of isoenzymes is still unclear (Hughes & Halestrap, 1981; Lau *et al.*, 1981; Paul & Adibi, 1982).

Branched-chain 2-oxoacidaemia is an interesting disorder in that the altered BCOAD function varies from family to family and even within a family. Alteration in the structural gene of any component of the complex could lead to the diseased state resulting from improper assembly of the complex,

Abbreviations used: BCOAD, branched-chain oxo acid dehydrogenase (EC 1.2.4.4); Ig, immunoglobulin; SDS, sodium dodecyl sulphate.

impaired uptake or processing of the nuclear-coded translation products by the mitochondria, or altered active-site chemistry.

To begin addressing some of these problems, here we demonstrate the production of antibodies in rabbits to highly purified bovine liver BCOAD and to the isolated E3 component. Using these antibodies we can identify BCOAD in a variety of tissues and species. This cross-reactivity demonstrates a highly conserved BCOAD structure among mammalian species. Furthermore, antibodies to the complex react with each subunit, allowing identification of subunit composition from various sources.

Materials and methods

All reagents were of the highest grade available and solutions were made in deionized water. $1\text{-}^{14}\text{C}$ -labelled 2-oxo acids were prepared from precursor amino acids as previously described (Elsas *et al.*, 1974). Protein concentration was determined by the method of Bradford (1976). A Gilford 250 recording spectrophotometer interfaced with an Apple II Plus microcomputer was used for spectral analysis.

Preparation of antiserum

Antibodies were produced in New Zealand White rabbits (Gilreath) by multiple subcutaneous injections of the protein in Freund's adjuvant (Difco). Injection schedules were as follows: pig heart lipoamide dehydrogenase (Sigma) was injected at a concentration of 0.75 mg every 2 weeks; in separate rabbits, 0.3 μg of bovine liver BCOAD prepared as previously described (Heffelfinger, 1983) was injected on 3 consecutive days, followed by 0.1 mg booster injections at monthly intervals. The Ig fraction was prepared from the antisera by precipitation with 50% of $(\text{NH}_4)_2\text{SO}_4$. Ig was stored in 50 mM-potassium phosphate, pH 7.0, at 25 mg/ml and -20°C .

Preparation of Fab fragments (Spiegelberg & Weigle, 1965)

IgG was prepared from the Ig fraction by DEAE-cellulose chromatography. After binding the Ig in 5 mM-potassium phosphate, pH 8.0, the IgG was eluted from the column with 35 mM-potassium phosphate, pH 8.0. Papain digestion of the IgG (1 mg of papain/100 mg of Ig) was stopped after 6 h at 37°C in 150 mM-potassium phosphate, pH 7.0, containing 0.01 M-cysteine hydrochloride and 2.0 mM-EDTA. The digest was dialysed against 10 mM-acetate buffer, pH 5.5. Fab fragments were separated from Fc fragments and undigested IgG by CM-cellulose column chromatography (Porter, 1959). SDS/polyacrylamide-gel electrophoresis of the unbound fraction showed the Fab fragment as a single protein band, with M_r 40 000.

Immunoelectrophoresis

Rocket immunoelectrophoresis was performed with a 1% agarose gel containing 2% Ig fraction and 1% Triton X-100. Electrophoresis took 15–18 h at 120 V and 4°C , in a Tris/borate buffer, pH 8.6. After electrophoresis the gels were washed consecutively in 0.2 M-NaCl, water and 12.5% trichloroacetic acid. Precipitated antigen–antibody complexes were stained with 0.0125% Coomassie Brilliant Blue G in 12.5% trichloroacetic acid.

Mitochondrial-inner-membrane preparation

Fibroblasts were washed in 0.25 M-sucrose/0.1 mM-EDTA/1 mM-potassium phosphate, pH 7.2, and centrifuged at 700 g for 5 min. The cell pellet was mixed with the same buffer at a ratio of 400 mg wet wt./10 ml of buffer. For every 10 ml of cell suspension, 25 μl of Protease VI (Sigma) (0.1 mg/ml) was added and digestion was allowed to proceed for 7 min at 0°C . The cells were homogenized in a glass/Teflon grinder and the volume was doubled with buffer. Whole cells and nuclei were removed by centrifugation at 700 g for 10 min, and the mitochondrial fraction was precipitated by centrifugation at 10 000 g for 10 min. This mitochondrial pellet was suspended in a minimal volume of buffer and the outer membrane was stripped by digitonin treatment (1 mg of digitonin/mg of mitochondrial protein). After 20 min exposure to the detergent, the reaction volume was doubled with buffer and the inner-membrane fraction pelleted by centrifugation at 15 000 g for 15 min.

Mitochondria from animal tissues were prepared by conventional methods of differential centrifugation of tissue homogenates in the sucrose/EDTA/phosphate buffer. Inner membranes were prepared with digitonin treatment as for fibroblast mitochondria, or a soluble fraction was made from the mitochondria by suspending the mitochondria in 20 mM-phosphate buffer, pH 6.5, and freezing at -70°C and then centrifuging the thawed solution at 30 000 g for 45 min.

Western blot (Burnette, 1981)

Protein samples in duplicate were resolved by electrophoresis on a SDS/10% polyacrylamide gel, and the gel was soaked for 30 min with two changes in 25 mM-Tris/glycine, pH 8.3, diluted with 0.2 vol of methanol. Electroblotting was done in an E-C Electroblot apparatus (E-C Apparatus Corp., St. Petersburg, FL, U.S.A.) on to nitrocellulose according to the manufacturer's instructions. Blotting took less than 4 h at 300 mA. After blotting, the nitrocellulose was soaked in 0.15 M-NaCl/0.05 M-Tris/HCl (pH 7.4) containing 1% gelatin (Sigma type III) and 0.1% Tween 20 (Medium I) for 60 min. Protein-impregnated nitrocellulose was cut into duplicate pieces and each was incubated for 1 h in

2 ml of bovine serum + 36 ml of 0.15 M-NaCl/0.05 M-Tris/HCl (pH 7.4) containing 0.1% Tween 20, 0.005 M-EDTA and 0.25% gelatin (Medium II). To each solution 50 μ l of specific or control antiserum was added, and the nitrocellulose strips were allowed to incubate overnight at room temperature. The blots were rinsed for a minimum of 3×10 min with Medium II and incubated for 2 h with 60 μ l of horseradish peroxidase-conjugated goat anti-(rabbit IgG) IgG and 300 μ l of bovine serum in 12 ml of Medium II. Each strip was then rinsed for 4×10 min with Medium II, and the peroxidase reaction was developed with freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (Sigma) (60 mg in 200 ml of 0.005 M-Tris/HCl, pH 7.5, +33 μ l of 30% H_2O_2). (**Care: this is a carcinogen.**) The colour reaction was stopped by rinsing in water.

Enzyme assays

BCOAD complex activity was measured by quantifying the formation of NADH by using the increase in A_{340} with time. Assay conditions have been described (Danner *et al.*, 1979), and the mixture contains, in 500 μ l: 30 mM-potassium phosphate, pH 7.5, 5 mM-dithiothreitol, 100 μ g of bovine serum albumin, 0.5 mM-CoA, 1 mM-NAD⁺, 0.2 mM-thiamin pyrophosphate, 0.2 mM-MgCl₂, 1–50 μ g of enzyme protein, and 0.1 mM oxo acid to start the reaction. When $^{14}\text{CO}_2$ was used to measure activity the reaction mixture was contained in 250 μ l and $1\text{-}^{14}\text{C}$ -labelled oxo acid served as substrate.

Results

Immunoglobulin from rabbits sensitized with bovine liver BCOAD specifically inhibited catalytic

activity of this enzyme complex when production of either CO_2 or NADH was used to quantify the function of the complex. As shown in Fig. 1(a), 50% inhibition of NADH production occurs at a protein ratio of 20:1 (Ig:BCOAD, w/w). This Ig fraction also inhibited purified rabbit liver BCOAD (kindly given by Dr. Ralph Paxton) at an identical protein ratio. The mechanism for inhibition does not depend on precipitation of antigen, since (1) Fab fragments made by papain digestion of Ig also inhibited BCOAD activity (50% inhibition at a protein ratio of 1 μ g of Fab: 1 μ g of BCOAD) and (2) inhibition was observed when mitochondrial inner membranes served as the enzyme source and $^{14}\text{CO}_2$ production was used to measure activity with 4-methyl-2-oxo-[1- ^{14}C]pentanoate as substrate. A protein ratio of 5:1 (Ig:membranes, w/w) resulted in 50% inhibition. Specificity of this Ig fraction was demonstrated when the activity of the structurally similar 2-oxoglutarate dehydrogenase complex in these mitochondria was inhibited by less than 25% at this same protein ratio with 2-oxo[1- ^{14}C]glutarate as substrate.

Antisera were also prepared against pig heart lipoamide dehydrogenase, the putative E3 of BCOAD as well as of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes. IgG fraction prepared from the sera was able to inhibit NADH production by BCOAD (Fig. 1b), suggesting that antigenic determinants similar to lipoamide dehydrogenase are in the E3 component of BCOAD complex. A 50% inhibition occurred at a protein ratio of 2.5:1 (IgG:BCOAD). E3 was isolated free of BCOAD E1 and E2 components by $(\text{NH}_4)_2\text{SO}_4$ elution of complex bound to calcium phosphate gel cellulose (Koike & Hamada, 1971;

Fig. 1. Inhibition of BCOAD by rabbit antisera

(a) ○, BCOAD activity in the presence of preimmune Ig; □, BCOAD activity in the presence of anti-BCOAD Ig. Activity was measured by NADH formation in a typical reaction mixture described in the text and containing 20 μ g of BCOAD protein. Addition of 0.1 mM-3-methyl-2-oxobutyrates was used to begin the reaction. Activity of 0.31 μ mol of NADH/min per mg of protein = 100%. (b) ○, BCOAD activity with preimmune sera; Δ, effect of anti-(pig heart lipoamide dehydrogenase) IgG. Reaction conditions were as in (a). Activity of 1.22 μ mol of NADH/min per mg of protein = 100%. No prior incubation with antisera was required for this inhibition.

Fig. 2. Ouchterlony double-diffusion analysis of bovine liver BCOAD and its separated component proteins. Pig heart lipoamide dehydrogenase antisera (100 μ g of protein/centre well) was made to react against bovine liver BCOAD (well 1; 16 μ g of protein), E3 extracted from BCOAD complex (well 5; 6.5 μ g of protein) and a mixture of the remaining proteins extracted from the complex (well 3; 3.5 μ g of protein). Reference pig heart lipoamide dehydrogenase is in wells 2, 4 and 6 (11 μ g of protein/well).

Fig. 3. Quantification by rocket immunoelectrophoresis of BCOAD in crude extracts of bovine liver and mouse liver mitochondria prepared by freeze-thaw disruption in 20mM-phosphate buffer, pH 6.5, containing 50mM-NaCl.

Wells 1 and 2 contain 36 and 18 μ g of protein respectively, solubilized from bovine liver mitochondria. Wells 3 and 4 contain 22 and 11 μ g of protein solubilized from mouse liver mitochondria. Wells 5 and 9, 6 and 10, 7 and 11, 8 and 12 contain 1.2, 0.8, 0.6 and 0.4 μ g of purified BCOAD protein respectively.

Fig. 4. Identification of BCOAD peptides in mitochondrial inner membranes resolved by SDS/polyacrylamide-gel electrophoresis.

After electrophoresis under reducing conditions, the proteins were transferred to nitrocellulose paper as described in the text. Detection of cross-reacting protein (lanes 1 and 4) was by diaminobenzidine staining of peroxidase-conjugated goat anti-(rabbit IgG) IgG. Lane 1 is 'blotted' mouse liver mitochondrial inner membrane and 4 the 'blotted' BCKD. Lane 5 is 'blotted' BCOAD after reaction with preimmune Ig. Lanes 2 and 3 are protein-stained duplicate gels of membrane and BCOAD. Arrows indicate detected lines, with corresponding M_r ($\times 10^{-3}$) of listed values.

Heffelfinger, 1983). Lines of partial identity can be shown by Ouchterlony double-diffusion precipitation between pig heart lipoamide dehydrogenase and E3 extracted from bovine liver BCOAD, as well as between BCOAD complex and pig heart lipoamide dehydrogenase (Fig. 2).

Anti-BCOAD Ig can be used to identify the antigen in crude mitochondrial extracts from other mammals. Freeze/thaw salt extracts of bovine and mouse liver mitochondria were quantified by rocket immunoelectrophoresis. Fig. 3 shows these extracts compared with bovine BCOAD standards. From this analysis, mouse liver contains 17 μ g of cross-reacting material per mg of protein in the crude mitochondrial extract.

Qualitative estimation of BCOAD antigen can be obtained by the technique of Western blotting. Mitochondrial-inner-membrane proteins prepared from various tissue sources when separated electrophoretically, transferred to nitrocellulose paper and

allowed to react with the anti-BCOAD Ig may be detected by using peroxidase-conjugated goat anti-(rabbit IgG) IgG and diaminobenzidine stain for peroxidase activity. Fig. 4 compares these reaction products from purified BCOAD with that found in mouse liver mitochondrial inner membranes. Each subunit reacts with the anti-BCOAD serum, allowing specific subunit identification from inner membranes of various sources.

Discussion

Antibodies specific for determinants on the multienzyme complex branched-chain 2-oxo acid dehydrogenase were produced in rabbits. In a similar manner, component proteins of the complex, as reported here for the E3 component, are also antigenic. Both sets of antibodies inhibit catalytic activity of native BCOAD. Our laboratory and others have presented data suggesting that the BCOAD complex is structurally and functionally similar to the well-characterized pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes and that all three complexes use a similar E3 flavoprotein (Pettit *et al.*, 1978; Heffelfinger, 1983). Sakurais *et al.* (1970) have shown that the E3 components of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes are structurally similar and functionally interchangeable. If a common lipoamide dehydrogenase functions in the three complexes, and it is present on the surface of these complexes, then the finding reported here that BCOAD-specific Ig partially inhibits 2-oxoglutarate dehydrogenase would be expected. Similarly, we were able to see weak cross-reactivity between this anti-BCOAD serum and partially purified rabbit liver pyruvate dehydrogenase complex (kindly given by Dr. R. Paxton) by rocket immunoelectrophoresis (results not shown). As expected, the IgG fraction against pig heart lipoamide dehydrogenase also cross-reacted with the rabbit liver pyruvate dehydrogenase complex when tested by this method (results not shown). These results support the fact that E3 is common to these three complexes.

The mechanism for inhibition of catalytic activity does not depend on a precipitating antigen-antibody complex. Fab fragments which do not allow the cross-linking necessary for precipitation will bind BCOAD and inhibit catalytic activity. Furthermore, inhibition by antibody was also seen when CO₂ production from branched-chain 2-oxo acids by mitochondrial inner membranes was measured. We have not been able to determine whether inhibition by the antibody is due to loss of substrate binding, electron transfer, or some other mechanism. Only when the individual enzyme activities can be quantified in the separated components can we begin to address this question.

The fact that antibodies to native BCOAD are able to cross-react with subunits separated by SDS/polyacrylamide-gel electrophoresis, as shown in Fig. 4, was somewhat unexpected, since some of the subunits, particularly the E2, are predicted to be buried in the complex. This finding suggests that all four subunits of BCOAD have immunogenic sites which remain antigenic after dissociation. The immunogenic sites on pig heart E3 are similar to the antigenic sites on E3 in native BCOAD, since binding and inactivation of the complex occurs with IgG raised to the pig heart enzyme. Further, the SDS/polyacrylamide-gel-electrophoresis-separated E3 in native BCOAD can be detected by the Western blot technique as a single cross-reacting band at the appropriate molecular weight of native E3.

Having demonstrated that these antibodies will cross-react with different species and tissues, both qualitative and quantitative measurements are possible. As seen with the peroxidase-stained Western blots, the four bands of the complex clearly stain. When applied to fibroblasts cultured from homozygous patients affected with branched-chain 2-oxoacidaemia, this technique can be used to identify which peptides in the complex might be absent or present with a different molecular weight. Only mitochondrial inner-membrane preparations are necessary, so small amounts of fibroblast cultures can be used. As antibodies to the individual subunits become available, quantification with rocket immunoelectrophoresis of the individual peptides will be possible. Currently we can quantify the amount of native enzyme in mitochondrial extracts of a number of tissues. Such quantification with the fibroblasts of branched-chain-2-oxoacidaemia patients will be of value only if gross changes have occurred.

Availability of these antibodies thus opens new avenues of investigation, which include screening fibroblast cell lines known to be deficient in BCOAD activity for BCOAD-subunit composition and generation of complementary-DNA probes for studying patient populations with inherited diseases affecting the complex.

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