Partial Purification and Characterization of Two Enzymes Involved in Isovaleric Acid Synthesis in *Clostridium bifermentans*

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Conversion of leucine to isovaleric acid by *Clostridium bifermentans* is achieved by the action of at least two enzymes. One is a transaminase producing α -ketoisocaproic acid, which was purified 30-fold from osmotic lysates of late-exponential phase cells by repeated chromatography on DEAE-Sepharose C16B and Sephacryl S300: this represented a 147-fold purification of activity found in sonically disrupted cells. This enzyme had an apparent molecular weight of approximately 190 000 and was composed of six identically sized sub-units (molecular weight $31\,000 \pm 1000$). Transamination required pyridoxal phosphate and pyruvate and was optimal at pH 8.6; the apparent K_m for leucine was 7.0 mM. Activity was totally inhibited by 1 mM-*p*-chloromercuribenzoate and partially inhibited by other thiol reagents. The second enzyme decarboxylated α -ketoisocaproic acid to form isovaleric acid and was also partially purified by chromatography on DEAE-Sepharose C16B and Sephacryl S300. It has an apparent molecular weight of 240 000 and required FAD and coenzyme A for activity; the K_m for α -ketoisocaproic acid was 4.2 mM and activity was optimal around pH 8.0. This enzyme was a flavoprotein with absorption maxima at 280, 320 and 400 nm, and a fluorescent maximum at 500 nm. The prosthetic group, FAD, dissociated from the protein during purification resulting in an inactive apoenzyme which was only partially re-activated by FAD. Activity was completely inhibited by several thiol reagents tested at 1 mM.

INTRODUCTION

Amino acid degradation in many species of anaerobic bacteria is often achieved by a mechanism known as the Stickland reaction, where oxidation and reduction reactions occur between one or more amino acids or non-nitrogenous compounds derived from amino acids (see review by Barker, 1981). Usually, one amino acid functions as a proton donor which is oxidatively deaminated and decarboxylated; reducing power generated in this reaction is consumed during reductive deamination of the amino acid proton acceptor. Although these reactions are of some metabolic significance, as they allow energy production during growth on protein hydrolysates, there have been relatively few studies on the enzymes involved in either the oxidative or reductive branches of this fermentation. Many of the schemes describing the mechanisms of these reactions are devised from data derived from cultural studies and conversion of substrates to products by whole cells. The notable exceptions involve glycine and proline reduction where the enzymes have been extensively purified (Seto & Stadtman, 1976; Tanaka & Stadtman, 1979; Turner & Stadtman, 1973), and oxidation of ornithine (Barker, 1981; Jeng *et al.*, 1974).

Leucine had normally been regarded as a proton donor in the Stickland reaction where it is converted to isovaleric acid (iV) (Barker, 1961; Nisman, 1954). However, cultural studies by Elsdon & Hilton (1978) indicated that leucine is also reduced to 4-methylvaleric (isocaproic) acid (iC). We have recently demonstrated (Britz & Wilkinson, 1982*a*) that washed cell suspensions of several species of proteolytic clostridia and *Peptostreptococcus anaerobius* convert leucine to iV

Abbreviations: iC, isocaproic acid; α kiC, α -ketoisocaproic acid; iV, isovaleric acid.

and iC within the one reaction whose stoichiometry is compatible with that of the Stickland reaction. Here, leucine acts as both proton donor and acceptor, so providing a unique system for studying the relationships between the oxidative and reductive branches of the Stickland reaction.

Leucine oxidation can be detected in cell-free extracts prepared by osmotic lysis or sonication (Britz & Wilkinson, 1983). Alternative methods of lysis of *Clostridium bifermentans* have been used to demonstrate that leucine oxidation is catalysed by at least one soluble enzyme [deaminating leucine to α -ketoisocaproic acid (α kiC)] and one membrane-associated enzyme (decarboxylating α kiC to iV) (Britz & Wilkinson, 1983). Both enzymes were solubilized by sonication. Reduction of leucine by this species was closely linked to the oxidative reaction and did not occur if the oxidative reaction was replaced by artificial proton donors such as dimercaptans, which have been used to detect glycine and proline reduction (Seto & Stadtman, 1976; Turner & Stadtman, 1973): this activity was also membrane-associated and was lost if cells were disrupted by sonication. Decarboxylase activity was stimulated by at least two dialysable factors (molecular weights < 3500), which were released during osmotic lysis and which were separated from activity when extracts were divided into soluble and particulate components. In addition to these yet unidentified factors, oxidation of leucine to iV required several cofactors including coenzyme A, FAD and pyridoxal phosphate with some dependence on pyruvate and NAD⁺ or NADP⁺. The oxidative system was sensitive to inhibition by oxygen but could be partially stabilized by reducing reagents. In the present communication, we describe the purification and some of the properties of two enzymes involved in leucine oxidation. This work has been presented in preliminary form (Britz & Wilkinson, 1982b).

METHODS

Organisms, media and growth conditions. *Clostridium bifermentans* strain MB49 was used throughout this work. Its isolation, identification and conditions of growth have been described previously (Britz & Wilkinson, 1982a). Late-exponential to early-stationary phase cultures were prepared using a 15% (v/v) inoculum from a stationary phase culture into proteose peptone broth pre-reduced in an anaerobic chamber under an atmosphere of 5% H₂, 10% CO₂ and 85% N₂. Cultures were set up in 400 ml bottles which were sealed in the chamber then removed for incubation at 37 °C for 3 to 4 h, unshaken.

Preparation of cell-free extracts. Cells were harvested by centrifugation (10000 g, 20 min, 4 °C) and washed in an equivalent volume of 0.05 M-Tris/HCl containing 100 mM-K₂HPO₄ and 1 mM-EDTA (TKE buffer, final pH 8.2). For osmotic lysis, cells were subsequently resuspended in 2.5% of the culture volume in TKE buffer plus 20% (w/v) sucrose; lysozyme, when used, was added at 0.5 mg ml⁻¹. The cell suspension was incubated at 37 °C for 10 min, when the cells were collected by centrifugation (3000 g, 10 min, 22 °C) and the supernatant fluid was discarded. The pellet was rapidly dispersed in an equivalent volume of ice-cold TKE buffer and held at 4 °C for 30 min. Particulate material was removed by centrifugation (12000 g, 30 min, 4 °C) and the supernatant (osmotic shock fluid) was retained. For preparation of sonic lysates, cells harvested from cultures by centrifugation were resuspended in 2.5% of the culture volume in PME buffer (0.05 M-sodium phosphate, pH 7.6, containing 10 mM-2-mercaptoethanol) and disrupted using an MSE 500-W ultrasonic disintegrator at 0.76 mA, output 2–3 for 1 min at 4 °C. Cell debris was removed by centrifugation as above. All buffers were degassed before use and cell-free extracts were held under a head-space of N₂.

Assay procedures. Conversion of leucine to α kiC, or α kiC to iV, was measured in 0.5 ml incubation mixtures containing the following (μ mol): MgCl₂, 1.5; K₂HPO₄, 10; sodium pyruvate, 1; ADP and AMP, 2 each; NAD⁺, NADP⁺, NADH, FAD, pyridoxal phosphate, lipoic acid, 0.2 each; coenzyme A, 0.05. Reaction mixtures were buffered at pH 8.2 using 40 mM-Tris/HCl and contained 5 μ mol of substrate (either leucine or α kiC). When assaying partially purified decarboxylase activity, incubation mixtures contained up to 100 μ l (0.3 mg protein) of crude osmotic shock fluid which had been treated at 100 °C for 10 min to inactivate inherent enzyme activities. This provided the small molecular weight factors which stimulated decarboxylase activity. Reactions were started by the addition of crude or partially purified enzyme containing up to 1 mg protein. Tubes were gassed with N₂, sealed and incubated for 30 min (deaminase) or 4 to 6 h (decarboxylase) at 37 °C. Reactions were terminated by the addition of 0.1 ml of 1.2 M-HCl (deaminase) or 50% H₂SO₄ (decarboxylase). The amount of iV produced was determined by GLC as described previously (Britz & Wilkinson, 1982a). α kiC was measured using an assay based on that described by Taylor & Jenkins (1966). Reaction mixtures contained 0.6 ml sample, 0.9 ml H₂O and 0.5 ml 2,4-dinitrophenylhydrazine reagent. This reagent contained approximately 15 mM-2,4-dinitrophenylhydrazine in

1.2 M-HCl. After 10 min at room temperature, the hydrazone derivative of α kiC was extracted into 2.5 ml cyclohexane, shaken for 20 s and the emulsion was separated by brief centrifugation. The upper phase (2 ml) was removed to a fresh tube and 0.75 ml 10% Na_2CO_3 was added, mixed and the emulsion separated by centrifugation. The lower layer (0.5 ml) was removed and 1.0 ml 1.0 M-NaOH was added before reading absorbance at 440 nm. Standards contained up to 0.6 μmol α kiC. Activity was calculated as the rate of production of iV or α kiC in $\mu\text{mol h}^{-1}$. Effects of inhibitors and pH on rates of reaction were tested as described previously (Britz & Wilkinson, 1982a).

Pyridoxal phosphate was assayed using the method of Wada & Snell (1961). Phenylhydrazine hydrochloride was prepared by neutralizing 10 ml phenylhydrazine with 6 M-HCl, collecting the precipitate by filtration, washing with diethyl ether and drying under vacuum.

Protein was determined by the Lowry method using crystalline bovine serum albumin as standard, or from absorbance at 280 nm.

Gel chromatography. Sephacryl S200 and S300 (Pharmacia) columns (2.5 by 45 cm) were equilibrated at 4 °C in PME buffer. Fractions (2.4 ml) were collected at 36 ml h^{-1} . Ion-exchange chromatography was performed using two successive columns of DEAE-Sephacryl C16B (Pharmacia), the first 1.6 $\times$ 45 cm and the second 1.2 $\times$ 30 cm. Both were initially equilibrated at 4 °C in PME buffer and 6 ml or 3 ml fractions were collected at 50 ml h^{-1} .

PAGE. Electrophoresis was performed according to the methods of Weber & Osborn (1969) or Laemmli (1970). Non-denaturing slab gels contained 6% (w/v) acrylamide and denaturing (SDS) gels contained 7.5% (w/v) acrylamide; stacking gels contained 3% (w/v) acrylamide. Samples contained up to 75 μg protein in 50 μl , and 10 μg of each standard protein was applied. Samples were applied to the gel using 10 mA and subsequently run at 28 mA at room temperature. Proteins were stained with 0.1% Coomassie brilliant blue in methanol/acetic acid/water (5:1:5, by vol.) for 10 min at 60 °C then initially destained for 1 h in 5% acetic acid and 7.5% methanol, at 60 °C. For determination of native molecular weights of proteins the following standards were used: apoferritin (450 000), catalase (240 000), bovine serum albumin (67 000), ovalbumin (43 000), β -lactoglobulin (36 500), trypsin inhibitor (soy bean) (21 500) and cytochrome *c* (12 400).

Purification of enzymes. Cells from 4 l were pelleted, washed in TKE buffer then subjected to the osmotic shock procedure described above. Shock fluids were filtered (0.45 μm pore size, Millipore), yielding about 100 ml extract, containing 2 to 5 mg protein ml^{-1} , then applied to the first DEAE-Sephacryl C16B column. After washing the gel with 200 ml PME buffer, a linear gradient of 0 to 0.5 M-NaCl in 600 ml PME buffer was applied. Fractions exhibiting deaminating activity were pooled, concentrated by ultrafiltration (PM 10 membrane, Amicon), then applied to a column of Sephacryl S300. Pooled active fractions were applied to the second DEAE-Sephacryl C16B column, the gel was washed with 120 ml PME buffer and then eluted using a 300 ml linear gradient of 0 to 0.25 M-NaCl. Active fractions were analysed by non-denaturing PAGE and selected fractions pooled. This material was concentrated by collodion finger to 3 ml and reapplied to the Sephacryl S300 column. After concentration (three- to fivefold), fractions were analysed by non-denaturing and denaturing PAGE.

Decarboxylase activity was partially purified from an extract of sonically disrupted cells from 4 l of culture prepared as described above. Extracts (50 ml, containing 10 to 15 mg protein ml^{-1}) were applied to the first DEAE-Sephacryl C16B column, washed with 300 ml PME buffer containing 0.1 M-NaCl, then eluted using a linear gradient of 0.1 to 0.4 M-NaCl in 600 ml PME buffer. Active fractions were pooled, diluted twofold in PME buffer and applied to the second DEAE column. This gel was washed with 120 ml PME buffer containing 0.1 M-NaCl and then a 300 ml linear gradient of 0.1 to 0.25 M-NaCl was applied. Active fractions were pooled and concentrated by collodion finger to 3 ml and applied to the Sephacryl S300 column. Decarboxylase activity, absorbance at 280, 400 and 450 nm and fluorescence were measured in all fractions.

Analytical procedures. Spectrophotometric measurements were made on a Zeiss PMQ II spectrophotometer and fluorescence readings on an Aminco Bowman Spectrophotofluorimeter (American Instrument Co.) in cells with 1 cm light paths. FAD was measured from fluorescence at 530 nm (excitation at 445 nm) using 0.1 or 0.01 mM-standard solutions in appropriate buffers. When reading fluorescence on protein-containing samples, these were treated with 1 mg trypsin ml^{-1} for 30 min at 37 °C, then boiled for 10 min and the precipitated protein removed by centrifugation (3000 *g*, 10 min). Samples were also deproteinated using trichloroacetic acid, as described by Kozioł (1971). To convert FAD to FMN, samples and controls were treated with phosphodiesterase (50 units, 37 °C, 1 h) then protein was removed as above. The pH was adjusted using 0.6 M-HCl to 7.3 and to 2.7 to 3.0, and fluorescence at these pH values was recorded. Ammonium ion concentrations were measured using an ammonium ion electrode (detecting as little as 1 p.p.m.) as described previously (Britz & Wilkinson, 1982a).

Chemicals. All substrates, cofactors and proteins used for molecular weight calibrations (with the exception of bovine serum albumin) were obtained from Sigma; bovine serum albumin was from the Commonwealth Serum Laboratories, Australia. Phosphodiesterase (mixed types I and II, Crotalid viper venom) was purchased from Calbiochem, and *p*-chloromercuribenzoate from BDH. 2,4-Dinitrophenylhydrazine was purchased from Ajax Chemicals (Melbourne, Australia). All other chemicals were of analytical grade and purchased from standard commercial sources.

Fig. 1

Fig. 2

Fig. 1. Separation of leucine deaminase and decarboxylase activities by gel filtration through Sephacryl S200. Composite diagram showing the relative elution of deaminase activity originating in osmotic shock fluid and decarboxylase activity from an extract prepared by sonication. ○, Decarboxylase activity assayed in the presence of heated osmotic shock fluid; —, A_{280} for the osmotic lysate; ●, deaminase activity. Two ml of extracts were applied to the column, equilibrated at 4 °C in PME buffer and fractionated as described in Methods. The elution volumes of the relevant molecular weight markers catalase (Cat.) and bovine serum albumin (BSA) are indicated.

Fig. 2. Separation of leucine deaminase and decarboxylase activities by ion-exchange chromatography on DEAE-Sephacryl C16B. Composite diagram showing the relative positions of leucine deaminase and decarboxylase activities eluting from DEAE-Sephacryl C16B. Extracts were prepared from the same culture then separately applied to the gel equilibrated initially in PME buffer at 4 °C. The gel was washed with PME containing 0.1 M-NaCl until A_{280} was negligible, then a gradient of 0.1 to 0.5 M-NaCl in PME buffer (total volume 600 ml) was applied. The column was fractionated as described in Methods and decarboxylase activity was assayed in the presence of heat-inactivated osmotic shock fluid. NaCl concentrations were determined from conductivity readings. ●, Deaminase; ○, decarboxylase; ▲, NaCl concentration; —, A_{280} for the fractionation of the osmotic shock fluid.

RESULTS

Separation of deaminating and decarboxylating activities by gel chromatography

Cell free extracts of *C. bifermentans* strain MB49 prepared by sonication contained enzymes which converted leucine to α kiC and α kiC to iV (Britz & Wilkinson, 1983). When sonic extracts were applied to Sephacryl S200 or DEAE-Sephacryl C16B, decarboxylating and deaminating activities were poorly separated (Figs 1 and 2). Hence deaminase was purified from osmotic shock fluid which lacked significant decarboxylase activity and had a higher specific activity for deamination than sonic lysates. The apparent molecular weight of the deaminase was approximately 190000 and that of the decarboxylase was approximately 240000 as determined by gel filtration on calibrated Sephacryl S200 and S300 columns.

Partial purification and properties of decarboxylase activity

Decarboxylase activity was partially purified by chromatography on two DEAE-Sephacryl C16B columns (NaCl gradients, 0.1 to 0.4 M and 0.1 to 0.25 M), then on Sephacryl S300. However, recovery from these columns was poor (usually < 50% of applied activity was recovered at each step) possibly due to the instability of the enzyme, which was related to oxygen sensitivity and uncoupling of a chromogenic prosthetic group. Activity from the first DEAE-Sephacryl column eluted in a single peak coincident with a fluorescent chromophore absorbing maximally at 400 nm (see below). Tubes containing maximum activity were pooled, applied to

Fig. 3. Relationship between fluorescence and decarboxylase activity. Cells from a 41 proteose peptone broth culture were disrupted by sonication and the cleared extract in PME buffer was applied to a column of DEAE-Sephadex (1.6 × 45 cm) equilibrated in PME buffer. After washing and fractionating using a gradient of 0.1 to 0.4 M-NaCl (600 ml), as described in Methods, the fractions containing decarboxylase activity were pooled and diluted in PME buffer then applied to a second column of DEAE-Sephadex (1.2 × 30 cm) equilibrated in PME buffer. The gel was washed using 100 ml PME buffer containing 0.1 M-NaCl, then a gradient of NaCl was applied from 0.1 to 0.25 M in a total volume of 300 ml: the start of the gradient corresponds to fraction 25. Decarboxylase activity (●) was assayed in the presence of heat-inactivated osmotic shock fluid. The following readings were recorded for each fraction: fluorescence detected at 500 nm (○); A_{280} (—); A_{400} (■); A_{450} (□).

the second DEAE-Sephadex column and eluted as described in Methods (Fig. 3). Activity eluted during the 0.1 to 0.25 M-NaCl gradient in a single peak which was coincident with a peak of protein, fluorescence, and absorbance at 400 nm: absorption at 400 nm was greater than that at 450 nm. A small amount of protein was eluted during washing with 0.1 M-NaCl, as was a large peak of fluorescent material which absorbed at 450 nm: absorption at 400 nm was less than that at 450 nm. This material was not retained during dialysis. Active fractions were pooled, concentrated by collodion finger and applied to Sephacryl S300; the chromophore was protein-associated during concentration and the effluent had no detectable absorption at 400 or 450 nm. Activity eluted from Sephacryl S300 in a single peak coincident with a peak of fluorescence (profile not shown). However, there was insufficient activity remaining at this stage to allow further purification, so that sub-unit structure of this enzyme was not determined.

The association between decarboxylase activity and a fluorescent chromophore absorbing at 400 nm suggested that this enzyme may be a flavoprotein. The absorption profile for decarboxylase activity eluting from the final Sephacryl S300 column is shown in Fig. 4. Absorption maxima occurred at 400, 320 and 280 nm. FAD and material washing through the second DEAE-Sephadex column absorbed at 450 and 380 nm. The fluorescence maximum for decarboxylase activity was 500 nm as compared with 530 nm for FAD (Fig. 5). Atomic absorption spectroscopy failed to detect iron in excess of levels found in buffer controls. However, the iron levels expected in the samples tested were at the limits of sensitivity for detection ($>1.0 \text{ mg l}^{-1}$). Pyridoxal phosphate was not detected in association with this enzyme.

To prove that the prosthetic group was FAD, samples from the final Sephacryl S300 column were deproteinated either by trypsin or trichloroacetic acid treatment (Koziol, 1971) and fluorescence was read after adjusting the pH to approximately 3.0 or to 7.3: FAD has a fluorescence maximum at the lower pH, whereas FMN has almost equal fluorescence intensity over a broad pH range and has greater fluorescence than FAD. FAD was converted to FMN by

Fig. 4

Fig. 5

Fig. 4. Comparison of absorbance spectra for decarboxylase (—) and FAD (---). Decarboxylase activity was partially purified by ion-exchange chromatography on DEAE-Sephacrose C16B, eluted firstly using a NaCl gradient of 0.1 to 0.5 M, then applied to a second column and eluted with a gradient of 0.1 to 0.25 M-NaCl. The pooled active fractions were concentrated by collodion finger and applied to Sephacryl S300. Absorbance at 260 to 600 nm was determined using material from fractions containing maximum activity, concentrating threefold by collodion finger.

Fig. 5. Fluorescence maximum for decarboxylase activity, with excitation at 445 nm (■) and 400 nm (□). FAD (0.01 mM) in PME buffer (○) was used as a control with excitation at 445 nm. Decarboxylase activity was purified and concentrated as described in the legend to Fig. 4.

Table 1. *Identification of the chromophore associated with decarboxylase as FAD*

Fluorescence at high and low pH values was measured for crude osmotic shock fluid and partially purified enzyme from the final stage of purification, following four types of treatment: no enzyme treatments; deproteinized using trypsin; deproteinized using trypsin, then phosphodiesterase-treated; phosphodiesterase treatment only. FAD was treated similarly. As there was little difference in fluorescence before and after trypsin treatment, selected results only are given.

Sample	Treatment	Fluorescence at:		Ratio of fluorescence pH 3.0/pH 7.3
		pH 7.3	pH 3.0	
Purified enzyme	None	0.015	0.023	1.5
	Trypsin	0.015	0.024	1.6
	Phosphodiesterase	0.043	0.036	0.8
FAD (0.1 mM)	None	2.43	9.30	3.8
	Phosphodiesterase	15.39	12.30	0.8
Crude osmotic shock fluid	Trypsin	0.562	0.655	1.2
	Phosphodiesterase	0.785	0.600	0.8
FAD + crude osmotic shock fluid	Trypsin	3.75	8.80	2.3
	Phosphodiesterase	9.45	9.20	0.9
Wash from 2nd DEAE column	Trypsin	2.90	2.65	0.9
	Phosphodiesterase	3.00	2.79	0.9

phosphodiesterase, then fluorescence at different pH values was determined. Samples of purified enzyme and crude osmotic shock fluid were treated similarly, with and without deproteinization. Results following trypsin or TCA treatments were identical and those for the

Table 2. *Cofactor requirements of leucine deaminase and decarboxylase activities*

Enzymes were partially purified from osmotic or sonic lysates by ion-exchange chromatography on DEAE-Sephadex.

Omitted cofactor	Enzyme activity*	
	Leucine→ α kiC	α kiC→iV†
K ₂ HPO ₄	85	100
MgCl ₂	99	96
ADP	108	109
AMP	89	112
ADP + AMP	90	88
Pyruvate	45	115
Coenzyme A	64	22
Pyruvate + Coenzyme A	32	17
NAD ⁺	62	114
NADH	116	108
NADP ⁺	116	124
NAD ⁺ + NADH	32	130
NAD ⁺ + NADP ⁺	NT	110
NAD ⁺ , NADH + NADP ⁺	50	122
FAD	54	13
Lipoic acid	132	109
Pyridoxal phosphate	17	95

NT, Not tested.

* Percentage of activity found in assays containing all cofactors. Data were obtained from duplicates which showed 10% variation between assays.

† Assayed in the presence of osmotic lysate.

Table 3. *Effects of potential inhibitors on partially purified leucine deaminase and decarboxylase activities*

Inhibitor	Concn (mM)	Percentage inhibition*	
		Leucine→ α kiC	α kiC→iV
Iodoacetate	2	47	91
Azide	10	0	91
Ferricyanide	1	45	95
Cyanide	1	0	55
Arsenite	1	30	81
Arsenate	1	0	9
Phenosafranine	1	16	0
Neutral red	1	0	16
Methyl viologen	1	6	24
Methylene blue	1	6	0
Metronidazole	1	0	0
2,4-Dinitrophenol	2	49	84
<i>p</i> -Chloromercuribenzoate	0.1	42	45
<i>p</i> -Chloromercuribenzoate	1	96	95

* Percentage of inhibition relative to incubation mixtures lacking inhibitors.

former are presented in Table 1. These results are consistent with the prosthetic group being FAD. However, fluorescence of FAD at pH 3.0 was 3.8 times that at pH 7.3, whereas the enzyme was only 1.6 times more fluorescent at pH 3.0 than at pH 7.3. This lower ratio for the enzyme may have been due to partial conversion of FAD to FMN during storage or due to quenching; FAD showed some decrease in this ratio when trypsin-treated in the presence of osmotic shock fluid. The fluorescent material which washed through the second DEAE-Sephadex column, and which had been stored at 4 °C before testing, had the same fluorescence

Fig. 6

Fig. 7

Fig. 6. pH optima for assay of leucine deaminase (a) and decarboxylase (b) activities. Enzymes were partially purified by DEAE-Sepharose C16B chromatography, then activity was determined in the following buffers; 0.1 M-acetic/acetate (○); 0.05 M-citrate/0.1 M-phosphate (●); 0.1 M-sodium phosphate (□); 0.1 M-Tris/HCl (■); 0.1 M-glycine/NaOH (△); 0.15 M-phosphate/NaOH (▲).

Fig. 7. Denaturing PAGE of partially purified leucine deaminase following rechromatography on Sephacryl S300 (Table 4). (a) 10 μg protein; (b) 75 μg protein. Molecular weight markers: bovine serum albumin (1); catalase (2); ovalbumin (3); cytochrome c (4).

Table 4. *Purification of leucine deaminase activity*

Details of the experimental procedures are given in Methods.

Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units mg ⁻¹)	Percentage recovery	Purification (fold)
Crude sonic lysate				0.156		
Crude osmotic lysate	100	248	321	0.773	100	1
First DEAE-Sepharose	60	209	62.4	3.35	84	4.3
First Sephacryl S300	35	138	20.9	6.60	56	8.5
Second DEAE-Sepharose	37	79.4	5.68	13.9	32	18
Second Sephacryl S300	25	55.6	2.42	23.0	22	30

before and after phosphodiesterase treatment and was likely to be FMN. Attempts to stabilize decarboxylase activity by running columns in PME buffers containing FAD did not improve recoveries.

Decarboxylase activity required coenzyme A and FAD (Table 2) and was significantly inhibited by iodoacetate, azide, ferricyanide, arsenite, 2,4-dinitrophenol and *p*-chloromercuribenzoate (Table 3). The enzyme was active over a broad pH range with maximum activity between 7.8 and 8.6 (Fig. 6). The apparent K_m for conversion of α kiC to iV was 4.2 mM.

Purification and properties of deaminating activity

The enzyme converting leucine to α kiC was purified 30-fold from osmotic shock fluid by repeated chromatography on DEAE-Sepharose C16B and Sephacryl S300 (Table 4). Activity eluted from the final column in a single peak coincident with a protein peak, and analyses by non-denaturing PAGE revealed one major protein band corresponding to activity detected following elution from these gels. Denaturing PAGE showed that the enzyme was probably composed of one type of sub-unit with a molecular weight of approximately 32 000 (Fig. 7). This suggests that the enzyme is a hexameric protein, as the apparent molecular weight of the native protein was 190 000 as determined by gel filtration. The purified protein did not absorb in the visible region and pyridoxal phosphate was not found in association with the protein purified under these conditions.

Deaminating activity in partially purified preparations required pyridoxal phosphate for activity (Table 2). Although there appears to be some requirement for NAD^+ , NADH or NADP^+ , omitting all of these did not eliminate activity. Omitting pyruvate, especially in the absence of coenzyme A, decreased activity, whereas lipoic acid appeared to inhibit activity to some extent.

Activity was also partially inhibited by iodoacetate, ferricyanide, arsenite and 2,4-dinitrophenol, and significantly inhibited by *p*-chloromercuribenzoate (Table 3). The pH profile for the activity was similar to that for the decarboxylase (Fig. 6). The apparent K_m for conversion of leucine to α kiC was 7.0 mM.

To determine if the conversion of leucine to α kiC proceeded by oxidative deamination (yielding free ammonium ions) or transamination (using pyruvate as the NH_4^+ receiving keto acid), ammonium ion levels were measured following incubation of leucine in the presence of purified enzyme and its required cofactors. Although $1.34 \mu\text{mol } \alpha\text{kiC ml}^{-1}$ was formed after several hours incubation, free ammonium ions were not detected in levels above those of the controls (including heat-inactivated enzyme and enzyme incubated in the presence of leucine but without cofactors).

DISCUSSION

Early schemes describing the mechanism of the Stickland reaction did not make it clear whether the oxidative branch of the reactions involved transamination or oxidative deamination (Barker, 1961; Nisman, 1954). More recently, Bader *et al.* (1982) suggested that the initial reaction is a transamination involving α -ketoglutarate, which is regenerated by the action of glutamate dehydrogenase. This scheme proposes that the reductive branch of the Stickland reaction is coupled to the oxidative branch, forming an α -keto acid by transamination, which is then hydrogenated to form the α -hydroxy acid (hence consuming one equivalent of NADH formed by glutamate dehydrogenase). Dehydration of the α -hydroxy acid to form an enoate and subsequent hydrogenation of the enoate (catalysed by enoate reductase, consuming a second equivalent of NADH) leads to formation of the aliphatic acid. All of the enzymes involved in this scheme have been demonstrated in crude extracts of several species of proteolytic anaerobes (Bader *et al.*, 1982; Nisman, 1954) or have been purified, in the case of enoate reductase (Bühler *et al.*, 1980). Our results indicate that transamination is the initial step in leucine dissimilation in *C. bifermentans*. The enzyme purified from osmotic lysates of strain MB49 required pyridoxal phosphate for activity and was probably a hexamer of similar sized sub-units. Production of α -kiC was not associated with production of free ammonium ions. There was no absolute requirement for NAD^+ (or NADP^+) for reactions catalysed by partially purified enzyme, although activity was less in the absence of all of NAD^+ , NADH and NADP^+ . The reaction was dependent on added pyruvate, though the ability of other α -keto acids to replace pyruvate was not tested. This transaminase from *C. bifermentans* shares properties with transaminases from both eukaryotic and prokaryotic cells. These are often oligomeric proteins (frequently dimers or tetramers) which function at high pH, are sensitive to inhibition by carbonyl and thiol reagents and whose reaction mechanisms require pyridoxal phosphate (see review by Braunstein, 1973). Although pyridoxal phosphate is usually bound to the enzyme, there are cases where it is only

loosely associated and is consequently required for activity. This appears to be the case for the leucine transaminase of *C. bifermentans* reported here.

Decarboxylation of α kiC to iV was catalysed by a membrane-associated enzyme which contained FAD as an essential component for activity: removal of this group during purification resulted in an inactive apoenzyme whose activity was only partially restored by addition of exogenous FAD. The molar ratio of FAD to protein for the most purified preparation of this enzyme was 0.7 to 1.0 based on a molecular weight of 240 000 for the enzyme. Many FAD-containing enzymes are oligomers with one FAD bound per active sub-unit, and frequently contain labile sulphur and sometimes iron (see Singer, 1976). Assuming the *C. bifermentans* enzyme is similar to these, the low ratio of FAD relative to protein is consistent with the low activity associated with the purified enzyme and the observed loss of flavin during purification. Activity was inhibited by thiol reagents including *p*-chloromercuribenzoate, arsenite and iodoacetate. Inhibition by several thiol reagents suggests, at least, the presence of an essential sulphhydryl group near the substrate-binding site. Although we failed to detect iron in association with this enzyme, we cannot eliminate the possibility that it is an iron-sulphur protein. Many of the thiol reagents tested (e.g. *p*-chloromercuribenzoate and iodoacetate) react with sulphhydryl groups and some (cyanide, especially at high pH, azide and *p*-chloromercuribenzoate) with sulphide of iron-sulphur clusters (Hewitt & Nicholas, 1963; Lovenberg *et al.*, 1963). Activity was not inhibited by chelating agents (Britz & Wilkinson, 1982*a*), but some iron-containing enzymes are not affected by chelating agents. Presumably the FAD is involved with electron transfer associated with decarboxylation but the fate of the reducing power *in vivo* is not known.

Decarboxylation also required coenzyme A, although ADP and AMP were not essential for activity. Energy generation associated with leucine dissimilation probably occurs via the initial formation of an acyl-coenzyme A derivative which, *in vivo*, is converted to the acyl-phosphate form which is directly associated with ATP generation (Nisman, 1954). In our assay system, the acyl-coenzyme A derivative may possibly rapidly hydrolyse to form free isovaleric acid, as detected by GLC. Membrane association for this decarboxylase would be convenient for both ATP production and for transfer of reducing power to the membrane-located leucine reductase system.

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