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Purification and Properties of Threonine Aldolase from *Clostridium pasteurianum*

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1. Threonine aldolase was purified about 200-fold in 10% yield from *Clostridium pasteurianum* and its properties were examined. The final preparation gave three bands after ionophoresis on polyacrylamide gel. 2. The purified enzyme was shown to produce glycine and acetaldehyde in stoicheiometric amounts from threonine. The reverse reaction was demonstrated qualitatively. 3. The enzyme has a broad pH optimum at 6.5–7.0. 4. The enzyme is highly specific for L-threonine. 5. The enzyme is completely inhibited by 1mM concentrations of hydroxylamine and semicarbazide. Activity is decreased to 20% of the original by treatment with cysteine plus mercaptoethanol; most of the loss is regained on incubation with pyridoxal phosphate. It is concluded that pyridoxal phosphate is a prosthetic group. 6. The relationship between velocity and substrate concentration is atypical but indicates a K_m value of 0.42mM. 7. The enzyme was demonstrated in several other strictly anaerobic bacteria.

In the preceding paper (Dainty & Peel, 1970) evidence was presented that the strict anaerobe *Clostridium pasteurianum* synthesizes glycine by way of aspartate and threonine and that the final step in this pathway is the cleavage of threonine to give glycine and acetaldehyde. Threonine aldolase (L-threonine acetaldehyde-lyase, EC 4.1.2.5), which catalyses this cleavage, has been demonstrated in mammalian tissues (Lin & Greenberg, 1954). It has been purified from rat liver (Malkin & Greenberg, 1964) and from sheep liver (Karasek & Greenberg, 1957), but in neither case was the activity of the final preparations very high. Threonine aldolase has not up to now been demonstrated unequivocally in micro-organisms. This paper reports the purification of the enzyme from *C. pasteurianum* and describes some of its properties; a preliminary report of part of the work has already appeared (Dainty, 1967).

MATERIALS AND METHODS

Micro-organisms

Growth and maintenance. Clostridium pasteurianum strain W5 (N.C.I.B. 9486, A.T.C.C. 6013) was maintained and grown in cultures of up to 1.5l as described by Dainty & Peel (1970). Larger quantities of cells required for the enzyme purification were obtained by growing 40l cultures in 9gal. or 10gal. stainless-steel kegs (Fairey Stainless, Stockport, Cheshire, U.K.) on the phosphate medium of Lovenberg, Buchanan & Rabinowitz (1963). Provision for flushing with gas was made as for the 1.5l cultures. The bulk of the medium was sterilized in the drum by first bringing it to 15lb/in^2 in the autoclave and then releasing the pressure rapidly; this caused the medium to boil and so gave a thorough mixing. The pressure in the autoclave was again raised to 15lb/in^2 and maintained at this level for 90min. When the pressure fell to atmospheric inside the autoclave, the drum was removed and cooled to approx. 30°C by running water, while H_2+CO_2 (95:5) was bubbled through the medium (30–40min). The drum was then inoculated with a 5% (v/v) inoculum as described for 1.5l cultures and incubated at 30°C.

The cultures were harvested with a Sharples Superspeed continuous centrifuge when gas evolution ceased. The cell paste was dried over conc. H_2SO_4 under vacuum at room temperature, without prior washing of the cells. The dried material was ground in a coffee-mill and the powder stored at −20°C until required.

Escherichia coli strain 4071 from the Department of Microbiology, University of Sheffield, U.K., was grown on the complex medium of London & Knight (1966). Aerobic cultures of 200ml were grown in a 2l conical flask in a shaking incubator (model G-25; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). Anaerobic cultures of 500ml were grown in 1l Florence flasks fitted with a gassing attachment (cf. *C. pasteurianum*). The bacteria were grown under H_2. Both aerobic and anaerobic cultures were grown overnight at 37°C until growth was adequate as judged visually.

Pseudomonas fluorescens strain KB1 of Kogut &

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Podoski (1953) was grown on the following medium (g/l): Evans peptone, 10; Marmite, 3; NaCl, 5; succinic acid, 15. The medium was adjusted to pH6.6 before autoclaving and 200ml cultures were grown aerobically as described for *E. coli*.

Streptococcus faecalis N.C.T.C. 6782 was grown on the complex medium of Takebe & Kitahara (1963) with glucose (20g/l) as the substrate. Cultures were grown aerobically and anaerobically as described for *E. coli*.

Six strictly anaerobic bacterial strains from the avian caecum (Table 5) were obtained from Dr E. M. Barnes (Barnes & Goldberg, 1965). Cultures (800ml) were grown on the following medium (g/l): Evans peptone, 10; Oxoid Lab-Lemco beef extract, 10; Oxoid yeast extract, 3; cysteine hydrochloride, 0.5; glucose, 5. The pH of the medium was adjusted to 7.2–7.4 before autoclaving. Cultures were grown anaerobically as for *E. coli* but with H_2+CO_2 (95:5) as gas phase.

Peptostreptococcus elsdenii and *Veillonella alcalescens* were grown as described by Dainty & Peel (1970). Dried-cell powders of *Clostridium septicum* N.C.I.B. 547 and *Selenomonas ruminantium* strain HD4 were from the laboratory stock of micro-organisms and had been stored at −20°C for at least 18 months.

Preparation of cell-free extracts. Ultrasonic extracts and extracts of dried cells other than *C. pasteurianum* were prepared as described by Dainty & Peel (1970).

Methods used in purification of the enzyme

Enzyme assay. Threonine aldolase activity was measured by adding an excess of alcohol dehydrogenase (EC 1.1.1.1) to remove the acetaldehyde produced and by following the oxidation of NADH.

$$\text{Threonine} \rightarrow \text{glycine} + \text{acetaldehyde}$$

$$\text{Acetaldehyde} + \text{NADH} \rightarrow \text{ethanol} + \text{NAD}^+$$

The reaction was carried out under purified N_2 in cuvettes fitted with a side arm as described by Somerville (1968) by using a Unicam SP. 800 spectrophotometer to measure the extinction at 340nm. The concentration of the enzyme preparation under test was chosen so that the decrease in E_{340} was not greater than 0.3/min and under these conditions 10 units of alcohol dehydrogenase provided an adequate excess.

The standard assay system contained in a total volume of 3ml 83mM-potassium phosphate buffer, pH7.0, 20μmol of L-threonine, 0.3μmol of NADH and 10 units of alcohol dehydrogenase. The test enzyme sample was originally placed in the side arm and tipped to start the reaction. The reaction was linear until the NADH was nearly exhausted. Controls without threonine showed negligible activity after the first 30s and no correction was applied.

Protein determinations. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard, except during the calcium phosphate gel treatment. In that case a more rapid method was required and the extinctions of the preparation at 260 and 280nm were measured with a Unicam SP. 500 spectrophotometer. The protein concentration was then derived by using a nomograph (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.) based on the extinction data of Warburg & Christian (1942).

Calcium phosphate gel. This was prepared as described by Keilin & Hartree (1938). After the final washing with water the gel was centrifuged (2000*g* for 5min) and resuspended in 10mM-potassium phosphate buffer, pH7.0, to give a suspension containing 10–15mg dry wt. of gel/ml of buffer. The suspension was stored at 0–5°C in a light-proof bottle for at least 1 month before use.

DEAE-cellulose column. DEAE-cellulose DE 11 (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) was prepared for use by suspension in 0.2M-NaOH, filtration on a sintered-glass funnel, washing with more 0.2M-NaOH until the effluent was colourless, and then washing with water until the effluent was free of alkali. The DEAE-cellulose was then suspended in 100mM-potassium phosphate buffer, pH7.0, in a beaker and allowed to settle for 1h. Any particles still suspended were decanted off with the buffer and the process was repeated until the supernatant was clear. The DEAE-cellulose was then suspended in the same buffer and allowed to stand at 0–5°C for at least 48h before use.

The following operations were then performed at room temperature. Dissolved gases in the DEAE-cellulose suspension were removed by evacuation and the suspension was poured into a glass column fitted with a capillary outlet, covered with a glass-wool plug. The DEAE-cellulose was allowed to settle while excess of buffer ran out through the capillary. More suspension was added until the required size of column (20cm×2cm diam.) was obtained. The column was then equilibrated by passing 500ml of 10mM-potassium phosphate buffer, pH7.0, transferred to the cold-room and allowed to stand overnight before use.

Dialysis. This was done as described by Dainty & Peel (1970).

Examination of the reaction products

Qualitative examination. The reaction vessel was a Conway microdiffusion unit and the outer chamber contained, in a total volume of 3ml, 90mM-potassium phosphate buffer, pH7.0, 20μmol of L-threonine and purified enzyme containing 0.1mg of protein. The centre well contained 1ml of saturated (approx. 25mM) 2,4-dinitrophenylhydrazine in 2M-HCl. After incubation at 30°C for 2h in air, the contents of the outer chamber were deproteinized by adding 0.3ml of 50% (w/v) trichloroacetic acid and centrifuging.

The amino acids present in the supernatant were examined by subjecting a 20μl sample to ionophoresis on paper at pH1.8, followed by chromatography in butanol–acetone–water; a second sample of supernatant was co-chromatographed with glycine in butanol–acetone–water (Dainty & Peel, 1970).

The hydrazone formed in the centre well was examined in two ways. (*a*) About half of the suspension was extracted with ethyl acetate and the organic layer was in turn extracted with 10% (w/v) Na_2CO_3 (Friedemann, 1957). Samples of the ethyl acetate layer were then chromatographed on paper with heptane–methanol (2:1, v/v) (Huelin, 1952), together with markers of the

authentic 2,4-dinitrophenylhydrazones from formaldehyde, acetaldehyde, propionaldehyde and butyraldehyde. (*b*) The remainder of the suspension was centrifuged to obtain crystals of the hydrazone, which were dried on filter paper and examined by a combination of g.l.c. and mass spectrometry. This part of the work was done in conjunction with Mr R. Self and Mr T. C. Grey of this Institute.

For g.l.c. the free carbonyl compound was first regenerated by grinding a few crystals to a fine powder with four times their weight of 2-oxoglutaric acid and introducing the mixture into a regeneration cell (Ralls, 1960). The regeneration cell was connected to the column of the gas chromatograph by a stainless-steel capillary; this capillary was in the form of a U, which was immersed in liquid O_2 (−183°C). The regeneration cell was heated to 270°C and the volatile compounds liberated were trapped in the capillary loop. The oxygen bath was then removed from the loop and the volatile material 'flashed' on to the column of a Pye Series 104 Temperature Programming Gas Chromatograph by electrical heating. The column was a coiled stainless-steel capillary (100ft. long × 0.015in. bore) coated internally with Triton X-305 (Koch–Light Laboratories Ltd.). Part of the effluent was fed to the flame ionization detector and the retention time measured. The remaining effluent was fed to an Edwards 60° mass spectrometer and the output from the detector of this instrument was displayed on a Direct Recording Ultraviolet Oscillograph (Southern Instruments, Camberley, Surrey, U.K.).

The reverse threonine aldolase reaction was investigated by incubating the following at 30°C for 1h under N_2 in an anaerobic cuvette in a total volume of 1.0ml: 75mM-potassium phosphate buffer, pH7.0, 10μmol of glycine, 10μmol of acetaldehyde and purified enzyme containing 50μg of protein. The mixture was deproteinized and examined for amino acids by ionophoresis at pH1.8 as for the forward reaction.

Quantitative examination. For quantitative measurement of the products the reaction was carried out as described in Table 2, in the vessel used by Knight, Wolfe & Elsden (1966) for the chemical degradation of threonine. After incubation the reaction was stopped by heating the vessel in a water bath at 90°C for 2min, while holding the rubber caps firmly in place. The vessel was then incubated at 40°C for 3h to allow the acetaldehyde to diffuse over into the centre well, where it was trapped by semicarbazide and determined spectrophotometrically (Burbridge, Hine & Schick, 1950).

Threonine was determined by oxidizing with excess of periodate (Aronoff, 1956). The periodate oxidation was done in a similar vessel to that used for the enzymic reaction. The main compartment contained, in a total volume of 3ml, 87mM-potassium phosphate buffer, pH7.0, 100μmol of sodium periodate and 0.2ml of the deproteinized reaction mixture. The centre well contained 1ml of 250mM-semicarbazide hydrochloride. After incubation at 40°C for 3h the acetaldehyde formed and trapped in the centre well was determined as above.

Glycine was determined by the ninhydrin method of Yemm & Cocking (1955) on the periodate-treated reaction mixture. Ammonia was first removed by adjusting to pH10 with 0.2M-KOH, boiling for 3min and bringing the pH to 5 with 0.2M-HCl.

Miscellaneous

Polyacrylamide-gel electrophoresis. The apparatus and experimental conditions used were as described by Davis (1964).

Reagents. Chemical reagents were as far as possible of analytical reagent grade. D- and L-Threonine, DL-allothreonine and 2-mercaptoethanol were from Koch–Light Laboratories Ltd. Citrate buffers were the appropriate citric acid–trisodium citrate mixtures and potassium phosphate buffers the appropriate KH_2PO_4–KOH mixtures. Other details were as in the preceding paper (Dainty & Peel, 1970).

RESULTS

Purification procedure

Extraction. Dried cells (20g) were suspended in 200ml of 10mM-potassium phosphate buffer, pH 7.0, from which dissolved gases had been removed by evacuation in a Buchner flask. The flask was then re-evacuated and incubated at 30°C for 45min with occasional shaking. All subsequent operations were performed at 0–5°C. Insoluble material was removed by centrifugation (15000*g* for 15min) and discarded.

pH5 *treatment.* The pH of the supernatant was adjusted to 5.0 by the dropwise addition of ice-cold 3M-acetic acid, when a heavy precipitate formed. The mixture was stirred continuously during the addition of the acid and for 1h thereafter. The precipitated material was removed by centrifugation (20000*g* for 15min) and discarded.

First ammonium sulphate treatment. Finely ground ammonium sulphate (0.24g/ml of supernatant) was now added gradually to the supernatant with continuous stirring to give 40% saturation. When the additon was complete, stirring was discontinued to avoid frothing and the mixture was allowed to stand for 30min before centrifuging (20000*g* for 15min). The precipitate was dissolved in approx. 25ml of 10mM-potassium phosphate buffer, pH7.0, and the resulting solution was dialysed for about 15h against 5l of the same buffer, with one change of buffer after 4h.

Calcium phosphate gel treatment. The protein in the dialysed preparation was determined and calcium phosphate gel (0.5mg dry wt. of gel/mg of protein) was added. The mixture was stirred slowly for 15min, then centrifuged (5000*g* for 5min) and the gel was discarded. The protein concentration of the supernatant was again determined as above, and more calcium phosphate gel was added (3mg dry wt. of gel/mg of protein). After 15min the mixture was centrifuged (5000*g* for 5min) and the supernatant was discarded.

The enzyme was eluted from the gel by suspension in 25ml of ice-cold 20mM-potassium phosphate

buffer, pH 7.0, and stirring slowly for 10 min. The mixture was centrifuged (5000*g* for 5 min), the supernatant retained and the elution procedure repeated a further three times.

Second ammonium sulphate treatment. The four eluates from the calcium phosphate gel were pooled and ammonium sulphate (0.39 g/ml of eluate) added as before to give 60% saturation. After standing for 45 min the preparation was centrifuged (20000*g* for 15 min), the precipitate was dissolved in 5 ml of 10 mM-potassium phosphate buffer, pH 7.0, and the solution dialysed against 1 l of the same buffer for approx. 15 h.

DEAE-cellulose treatment. The dialysis residue was applied to a column of DEAE-cellulose and washed in with 2 ml of the 10 mM buffer. After the washings had entered the column, 100 ml of the same buffer was passed through at a flow rate of 2 ml/min and 3 ml fractions were collected. Unretarded colourless protein containing the aldolase activity was eluted in fractions 5–8, while a yellowish band remained adsorbed at the top of the DEAE-cellulose column.

Third ammonium sulphate treatment. The four active fractions were pooled and the procedure of the second ammonium sulphate treatment repeated except that the dialysis was stopped after 4 h. The resulting dialysis residue was used for all the experiments described below and is referred to as 'purified enzyme'.

Purification data from a representative run are given in Table 1. The purification was repeated several times giving similar results, with overall purifications between 160- and 210-fold and recoveries of enzyme units between 8 and 15%. In one experiment a better purification (300-fold) was obtained by adding ammonium sulphate to 50% saturation rather than 60% in the third ammonium sulphate precipitation. However, only 10% of the activity of the DEAE-cellulose eluates was recovered in this highly purified fraction and this modified procedure was not therefore adopted for general use.

Properties of the purified enzyme

Purity. A sample of the purified enzyme was subjected to ionophoresis on polyacrylamide gel. On staining the gel three protein bands were revealed, indicating that the enzyme preparation was not homogeneous.

Stability. Solutions of the enzyme were stored at 0–5°C. Under these conditions the activity slowly decreased to 50% of the original (250 units/mg of protein) after 10 days. Experiments with purified enzyme preparations were all carried out within a few days of preparation.

Products of the enzymic reaction. The products resulting from the action of the purified enzyme on threonine in the absence of NADH and alcohol dehydrogenase were investigated as described in the Materials and Methods section. The two-dimensional procedure for amino acids showed only one ninhydrin-positive spot besides threonine. This corresponded in position to glycine and co-chromatography in the single-dimensional system confirmed this identification.

The 2,4-dinitrophenylhydrazone from the volatile products had the same mobility as that of acetaldehyde when tested by paper chromatography. Examination of the hydrazone under the microscope, however, revealed two different types of crystals. As it appeared impractical to use the melting point as a means of identification, the free carbonyl compound was regenerated and examined

Table 1. *Purification of threonine aldolase from dried cells of* C. pasteurianum

The starting material was 20 g of vacuum-dried cells of *C. pasteurianum*. The unit of activity is the amount of enzyme required to oxidize 1 μmol of NADH/h in the standard assay. All protein values were obtained by the method of Lowry *et al.* (1951).

Stage of purification	Volume (ml)	Activity (units/ml)	Total units	Protein (mg/ml)	Specific activity (units/mg)	Recovery of activity (%)	Purification
Extract of dried cells	174	21.4	3730	17	1.3	100	1
Supernatant from pH 5 treatment	173	21.5	3540	11	1.9	95	1.5
Dialysis residue after first $(NH_4)_2SO_4$ treatment	31	85	2630	13	6.5	71	5.2
Pooled eluates from calcium phosphate gel	100	21	2100	0.62	34	54	27
Dialysis residue after second $(NH_4)_2SO_4$ treatment	5	261	1305	4.4	59	35	47
Pooled fractions from DEAE-cellulose	21	33	698	0.18	183	19	144
Dialysis residue after third $(NH_4)_2SO_4$ treatment	5	78	392	0.29	269	11	214

Table 2. *Stoicheiometry of the threonine aldolase reaction*

The reaction vessel contained, in a total volume of 5 ml, 95 mM-potassium phosphate buffer, pH 7.0, approx. 21 μmol of L-threonine and purified enzyme containing 0.2 mg of protein. In addition, the centre well contained 1.0 ml of 250 mM-semicarbazide hydrochloride. Incubation was carried out in air at 30°C for 1 h. Initial values were determined on a duplicate unincubated mixture.

	Amount (μmol)		
	Initial	Final	Change
Threonine	21.1	14.8	−6.3
Glycine	0	6.0	+6.0
Acetaldehyde	0	5.8	+5.8

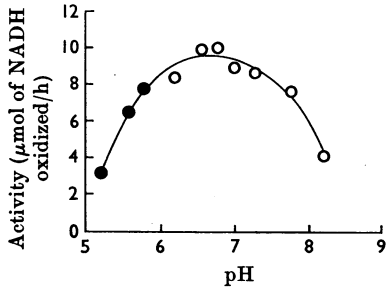

Fig. 1. Effect of pH on the activity of threonine aldolase. Each reaction mixture contained 83 mM-citrate or -potassium phosphate buffer of the pH indicated and purified enzyme containing 0.06 mg of protein. Other details were as for the standard assay. ●, With citrate buffer; ○, with phosphate buffer.

by g.l.c. and mass spectrometry. The g.l.c. revealed only one peak with a retention time (112 s) corresponding to that of authentic acetaldehyde. When this peak was examined in the mass spectrometer, the fragmentation pattern and the relative intensities of the parent ion and its fragments were identical with those for acetaldehyde, within the experimental error of the apparatus. Acetaldehyde is therefore a product of the enzymic reaction and is the sole volatile carbonyl compound formed.

Stoicheiometry of the reaction. When the enzymic reaction in the absence of NADH and alcohol dehydrogenase was examined quantitatively, only one-third of the threonine was utilized, but this gave rise to equivalent amounts of glycine and acetaldehyde (Table 2). The enzyme added was sufficient to metabolize all of the threonine present within the incubation period (based on its specific activity in the standard assay). The failure of the reaction to reach completion is attributed to the attainment of an equilibrium or to inactivation of the enzyme, possibly by the acetaldehyde formed.

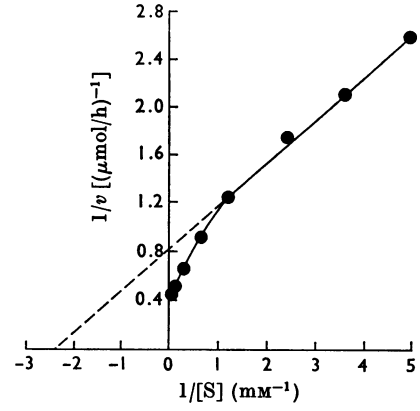

Fig. 2. Lineweaver–Burk plot for threonine aldolase. Reaction mixtures contained purified enzyme containing 0.04 mg of protein and the substrate concentration indicated. Other details were as for the standard assay.

Reverse reaction. This was demonstrated qualitatively by incubating the purified enzyme with acetaldehyde plus glycine as described in the Materials and Methods section. Ionophoresis of the products showed a ninhydrin-positive spot migrating as threonine and well separated from the glycine spot.

Effect of pH *on enzymic activity.* The pH optimum of the reaction was in the range 6.5–7.0 (Fig. 1).

Substrate specificity. When D-threonine replaced L-threonine in the standard spectrophotometric assay no measurable change in extinction was observed (an activity 2% of that with L-threonine would have been detectable). The two separate isomers of allothreonine were not available, but no activity was detected with racemic DL-allothreonine. Activity was also tested qualitatively under the conditions used to examine the products from L-threonine. With L-threonine a crystalline 2,4-dinitrophenylhydrazone appeared after 10 min of incubation, but with D-threonine and DL-allothreonine no hydrazone was detected even after 1 h. After 48 h, however, DL-allothreonine did give rise to a hydrazone, which was chromatographically identical with that of acetaldehyde. Neither serine nor homoserine was active in either of the above tests, with purified enzyme or crude ultrasonic extract. The threonine aldolase of *C. pasteurianum* is therefore highly specific for L-threonine.

Determination of the Michaelis constant. The effect of L-threonine concentration on the velocity of the reaction was determined by using the spectrophotometric assay system and the results were plotted according to the method of Lineweaver & Burk (1934). A typical result is shown in Fig. 2.

The relationship between $1/v$ and 1[S] is not linear, the reaction rates being disproportionately large at high substrate concentrations. The experiment was repeated several times and with different enzyme preparations, with similar results in all cases. These observations suggest that, under the conditions employed, threonine does not obey Michaelis–Menten kinetics over the whole range of substrate concentrations used. Such curvature of the graphs can be interpreted in terms of activation by excess of substrate, as was done for similarly curved plots for the α-chymotrypsin-catalysed hydrolysis of certain acylated glycine esters (Wolf & Niemann, 1959; Wolf, Wallace, Peterson & Niemann, 1964). As pointed out by Almond & Niemann (1960), this phenomenon can be simulated by undercorrection for the breakdown of substrate in the absence of enzyme or undercorrection for the enzyme blank. The latter possibility has been shown to be the probable reason for the curved plots for the α-chymotryptic hydrolysis of glycine esters by Ingles & Knowles (1966). In the present case neither of these explanations appears to be feasible, since under the conditions of the assay there is no spontaneous cleavage of the threonine molecule and all rates were corrected for the small oxidation of NADH that occurred in the absence of threonine. This being so, the points on the graph obtained at low substrate concentration were regarded as being the more reliable and a value for K_m was obtained by extrapolating a line drawn through these points back to the 1/[S] axis in the normal manner. The values obtained from four different determinations were 0.43mM, 0.40mM, 0.44mM and 0.41mM.

Prosthetic group. At no stage in the purification of the enzyme or in crude cell-free extracts was any stimulation of enzymic activity observed on the addition of mM-pyridoxal phosphate to the assay mixture. However, pyridoxal phosphate is often too tightly bound to the protein molecule for such stimulation to be demonstrated easily.

Several enzymes containing pyridoxal phosphate have been shown to be highly sensitive to inhibition by carbonyl reagents, which would be expected to react with the aldehyde group of pyridoxal phosphate, e.g. threonine aldolase from rat liver (Malkin & Greenberg, 1964) and threonine deaminase from *Neurospora crassa* (Yanofsky & Reissig, 1953). The effect of incubating purified threonine aldolase with hydroxylamine or semicarbazide was therefore investigated. Complete inhibition was obtained with 0.5mM-hydroxylamine and mM-semicarbazide (Table 3). These concentrations are of the same order as those reported by Malkin & Greenberg (1964) for the inhibition of rat liver threonine aldolase. These authors obtained reactivation of their enzyme preparation by incubating the inhibited enzyme with pyridoxal phosphate. With the threonine aldolase from *C. pasteurianum* no such reactivation was observed after incubating any of the inhibited enzyme preparations with mM-pyridoxal phosphate for 2h at room temperature. Resolution of pyridoxal phosphate from enzyme molecules has been achieved in several cases. For example, Schirch & Mason (1962) found that pyridoxal phosphate could be resolved from serine hydroxymethyltransferase by treatment with cysteine and concluded from spectral data that this was due to the formation of the thiazolidine compound of pyridoxal phosphate. Dupourque, Newton & Snell (1966), studying serine dehydrase, used a modified procedure involving dialysis of the enzyme against a solution of mercaptoethanol and cysteine. The mercaptoethanol was added in an attempt to keep the enzyme in its native form after removal of the pyridoxal phosphate. A sample of purified enzyme from *C. pasteurianum* was treated as described by Dupourque *et al.* (1966) and the effect of subsequent incubation with pyridoxal phosphate was examined. With two preparations tested the cysteine treatment decreased the activity to 20 and 25% of the original activities of the untreated preparations (Table 4). Subsequent treatment with pyridoxal phosphate for 5h restored the activities to 82 and 70% respectively of the original. No further

Table 3. *Inhibition of threonine aldolase by carbonyl reagents*

Purified enzyme containing 0.04mg of protein was incubated in air at 30°C with the inhibitor indicated for 5min, in a final volume of 1.0ml. The preparations were then dialysed against 10mM-potassium phosphate buffer, pH7.0, for 8h with three changes of buffer, and the activity was assayed.

Inhibitor	Concentration (mM)	Specific activity (units/mg of protein)	Inhibition (%)
None	—	151	0
Hydroxylamine	0.1	40	74
	0.5	4	97
Semicarbazide	1.0	0	100

increase in activity was observed by increasing the incubation period to 8h.

Solutions of purified threonine aldolase from *C. pasteurianum* were colourless and did not exhibit any absorption peaks other than the one at 278nm associated with proteins. Many enzymes containing bound pyridoxal phosphate exhibit a pH-independent absorption maximum in the region 410–430nm, which is due to the formation of a Schiff base between the aldehyde group of pyridoxal phosphate and an amino group on the enzyme molecule, e.g. rat liver threonine aldolase (Malkin & Greenberg, 1964). Some other pyridoxal phosphate-containing enzymes, such as aspartate aminotransferase (EC 2.6.1.1), exhibit an absorption maximum at 415–430nm in the pH range 4–5, whereas at higher pH values the peak shifts to the 330–365nm region, which is interpreted as indicating differences in the mode of binding of the prosthetic group (e.g. Schirch & Mason, 1962). While such features may constitute evidence for the presence of pyridoxal phosphate, their absence does not necessarily imply the absence of this prosthetic group. On balance, therefore, the present results indicate that the threonine aldolase of *C. pasteurianum* contains pyridoxal phosphate as a prosthetic group.

Table 4. *Resolution of pyridoxal phosphate from threonine aldolase*

Purified enzyme containing 0.3mg of protein in 1ml of 10mM-potassium phosphate buffer, pH7.0, was dialysed for 48h against 500ml of the same buffer containing 0.04M-L-cysteine and 0.02M-mercaptoethanol. After this thiol treatment the preparation was dialysed against 500ml of the phosphate buffer without thiols for 16h with three changes of buffer. The preparation was then incubated for the times indicated with mM-pyridoxal phosphate. Dialyses and incubation were at 0°C. Samples (0.1ml) were taken for assay as required.

Stage of treatment	Specific activity (units/mg of protein) Expt. 1	Expt. 2
Initial preparation	212	175
After thiol treatment	44	—
After final dialysis	44	44
After incubation with pyridoxal phosphate:		
for 5min	77	—
for 1h	135	—
for 5h	174	123

DISCUSSION

The present work provides unequivocal evidence for the presence of a specific threonine aldolase in *C. pasteurianum*. This enzyme has only recently been demonstrated in bacteria (Dainty 1967; Morris, 1969), although its presence was suggested earlier to explain the formation, in an auxotroph of *E. coli*, of a small amount of glycine from glucose by a route not involving serine as an intermediate (Simmonds & Miller, 1957). In *C. pasteurianum* the enzyme is constitutive and both tracer and enzymic evidence indicate that it is concerned in the biosynthesis of glycine from glucose via threonine (Dainty & Peel, 1970). The reverse reaction, i.e. the formation of threonine from glycine, could be demonstrated with pure preparations, but the tracer evidence shows that it is not significant in growing cells. The fate of the acetaldehyde formed

Table 5. *Threonine aldolase activity in different bacteria*

Assays were done on ultrasonic extracts, except with *C. septicum* and *S. ruminantium* where extracts of dried cells were used. The range of specific activities with replicate extracts is given. +, Enzyme demonstrated qualitatively although a quantitative value was not obtained.

Organism	Specific activity (units/mg of protein)
Clostridium pasteurianum	1.3–1.9
Selenomonas ruminantium	2–4
Caecal anaerobes strain EBF 61/42	7–10
strain EBF 58/74	0.4–0.8
strain EBF 61/60B	0.3–0.9
strain EBD 1/4a	0.2–0.4
strain EBF 59/96	0.2–0.6
strain Au 21/27	0
Clostridium septicum	+
Peptostreptococcus elsdenii, *Escherichia coli* (aerobic and anaerobic), *Pseudomonas fluorescens*, *Streptococcus faecalis* (aerobic and anaerobic) and *Veillonella alcalescens*	0

when glycine is produced from threonine has not been investigated in detail. Since extracts of *C. pasteurianum* were able to reduce acetaldehyde at the expense of NADH or NADPH it is possible that it may be converted into ethanol and excreted as an end product.

In a survey of several bacteria threonine aldolase was found in several anaerobic species (Table 5), and two of these (*S. ruminantium* and strain EBF 61/42) gave crude extracts with considerably higher activities than *C. pasteurianum*. This suggests that the biosynthesis of glycine via threonine may be common among anaerobes. On the other hand, it is possible that threonine aldolase may not be confined to a biosynthetic role in these organisms. It might also serve to initiate the catabolism of threonine as an alternative to the established route in which 2-oxobutyrate is first produced by threonine dehydratase (EC 4.2.1.16) (see, e.g., Barker, 1961).

In the present work threonine aldolase was not found in any of the aerobic organisms examined. However, Morris (1969) has obtained evidence that the enzyme is present in *Pseudomonas* species that are also able to oxidize acetaldehyde to acetyl-CoA. These organisms thus appear to possess a route from threonine to acetyl-CoA plus glycine that is different from the previously established aerobic route in *Arthrobacter*, via aminoacetoacetate (McGilvray & Morris, 1969).

The threonine aldolase of *C. pasteurianum* appears to contain pyridoxal phosphate as prosthetic group and in this respect resembles the enzyme from rat, sheep or rabbit liver (Malkin & Greenberg, 1964; Karasek & Greenberg, 1957; Schirch & Gross, 1968). It differs from the mammalian enzymes, however, in specificity. Whereas the bacterial enzyme attacks DL-allothreonine at no more than 2% of the rate with L-threonine, the mammalian enzymes attack DL-allothreonine more rapidly than L-threonine. Further, the K_m of 0.42mM for the bacterial enzyme with L-threonine compares with values of 20mM and 40mM for the rat- and rabbit-liver enzymes. Schirch & Gross (1968) obtained evidence that the threonine aldolase of rabbit liver is identical with serine hydroxymethyltransferase and observed that while the latter activity requires tetrahydrofolate, the former does not. The activity of the bacterial enzyme towards serine has not been tested in the presence of tetrahydrofolate. It is nevertheless clear that the threonine aldolase of *C. pasteurianum* has properties distinct from the mammalian enzymes.

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