Purification and Characterization of the Class-II D-Fructose 1,6-Bisphosphate Aldolase from *Escherichia coli* (Crookes' Strain)

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A new form of the class-II D-fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) of *Escherichia coli* (Crookes' strain) was isolated from an extract of glycerol-grown bacteria. It has a higher molecular weight (approx. 80000) than previous preparations of the enzyme and closely resembles the typical class-II aldolase from yeast in size and amino acid composition. On the other hand, its kinetic behaviour is not typical of a class-II aldolase. The enzyme has no requirement for thiol compounds either for stability or activity, added K⁺ ions have no effect, and the optimum pH for the cleavage activity is unusually high. The class-II enzymes from the prokaryote *E. coli* and the eukaryote yeast show no immunological identity. However, the similarity of their structures suggests that they have evolved from a common ancestor.

D-Fructose 1,6-bisphosphate aldolases (EC 4.1.2.13) can be divided into two classes on the basis of their catalytic and structural properties (Rutter, 1964). The reaction catalysed proceeds via a carbanion intermediate, and the two classes of enzyme differ primarily in the type of electron sink used to stabilize this intermediate. In class-I aldolases, typified by that from rabbit muscle, a protonated ketimine formed between the substrate C-2 carbonyl group and the N⁶-amino group of an active-site lysine residue is presumed to have this function (Horecker *et al.*, 1963; Model *et al.*, 1968). On the other hand, the aldolases of class II, typified by yeast aldolase, probably employ a bivalent transition-metal ion as an electron sink at the active site (Rutter, 1964; Mildvan *et al.*, 1971).

The typical class-I aldolases of plants and animals have received considerable attention and much is known about their structure and catalytic mechanism [for reviews see Morse & Horecker (1968) and Horecker *et al.* (1972)]. These eukaryote enzymes are invariably tetrameric, and amino acid-sequence studies indicate that they probably all belong to a single family of related proteins. In contrast, the relations of the commonly dimeric class-II enzymes remain a matter for conjecture. These less-well-studied aldolases are found both in the eukaryotic

green algae and fungi, and in the prokaryotic blue-green algae and bacteria. Their structural and catalytic properties are very diverse, and it is by no means certain that they are all evolutionarily related. The enzyme from the eukaryote yeast *Saccharomyces cerevisiae* has received the most attention. It is dimeric, has a mol. wt. of approx. 80000, and contains two tightly bound Zn²⁺ ions per molecule (Harris *et al.*, 1969; Richards & Rutter, 1961). The best-studied prokaryote aldolase is probably that from the thermophilic bacterium *Bacillus stearothermophilus*. This dimeric enzyme also contains tightly bound zinc, but is somewhat smaller than the yeast enzyme, having a mol. wt. of approx. 61000 (Sugimoto & Nosoh, 1971; Jack, 1973). In addition, it differs from most class-II aldolases in that its Fru-1,6-P₂-cleavage activity is not enhanced by added K⁺ ions, nor does the enzyme require the presence of thiol compounds for stability or catalytic activity (Hill *et al.*, 1976).

In the present paper we describe the detailed characterization of a class-II aldolase from another prokaryote, the mesophilic bacterium *Escherichia coli*. A previous report described the isolation from *E. coli* of a dimeric class-II aldolase (aldolase 2) with a mol. wt. of approx. 70000 (Stribling & Perham, 1973). The source of that enzyme was a bacterial extract that had received prior treatment with EDTA and protamine sulphate in the purification of 2-oxo acid dehydrogenase multienzyme complexes. The danger of proteolysis of the enzyme was noted at the time. We show here that a higher-molecular-weight form of aldolase 2 can in fact be isolated from bacterial extracts under suitable conditions. It is probable that this is the form of the enzyme *in vivo*.

Abbreviations used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Fru-1,6-P₂, fructose 1,6-bisphosphate; SDS, sodium dodecyl sulphate.

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Materials and Methods

Materials

Glass-distilled water and A.R. grade reagents were used except where otherwise stated. Urea was recrystallized from ethanol before use, and iodoacetic acid was twice-recrystallized from *n*-heptane. Dimethyl suberimidate dihydrochloride was kindly given by Dr. J. R. Coggins. Fru-1,6- P_2 (trisodium salt), purchased from C. F. Boehringer und Soehne, Mannheim, Germany, was repurified by chromatography on DEAE-cellulose as described by Webster *et al.* (1976), before use in kinetic studies. The repurified material gave a single spot when stained for carbohydrate (Trevelyan *et al.*, 1950) after high-voltage paper electrophoresis at pH 3.5. It was assayed by using rabbit muscle aldolase in the standard coupled assay described below. NADH (disodium salt), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, and coupling enzymes (a mixture of triose phosphate isomerase and α -glycerol phosphate dehydrogenases, 1:10, w/w) were also obtained from C. F. Boehringer und Soehne. Bovine serum albumin was from Armour Pharmaceuticals, Eastbourne, Sussex, U.K., and egg-white lysozyme chloride (grade I) was the product of Seravac Laboratories, Maidenhead, Berks., U.K. DEAE-cellulose was obtained from W. and R. Balston Ltd., Maidstone, Kent, U.K., and Sephadex G-150 was purchased from Pharmacia, Uppsala, Sweden. Hydroxyapatite was made by the method of Tiselius *et al.* (1956). Polyamide t.l.c. sheets were purchased from B.D.H. Chemicals Ltd., Poole, Dorset, U.K.

Methods

Enzyme assays. Two different methods of assaying aldolase activity were used. A colorimetric assay with 2,4-dinitrophenylhydrazine, described by Stribling & Perham (1973), was used during the purification of aldolase 2. One unit of aldolase activity measured in this assay was defined as that which caused a change in A_{540} of 1.0 after an enzymic-reaction period of 20 min. A coupled enzymic assay was used for more accurate kinetic studies (Blostein & Rutter, 1963). This assay was routinely performed at 30°C in a 1 ml final volume of 80 mM-glycylglycine buffer adjusted to the appropriate pH with NaOH. The assay buffer contained Fru-1,6- P_2 (5 mM), NADH (0.141 mM) and coupling enzymes (0.02 mg/ml). Further additions of coupling enzymes were made in assays performed at high pH or in the presence of various additives, to ensure that their own turnover was not rate-limiting. The decrease in A_{340} after addition of aldolase (10 μ l) was recorded by using a Hilger and Watts Uvichem mark 2 digital-model spectrophotometer coupled to a Servoscribe 1s chart recorder. One international

unit (i.u.) of aldolase activity is that causing the oxidation of 2 μ mol of NADH/min in this assay system. The molar extinction coefficient for NADH was taken as 6220 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ (Horecker & Kornberg, 1948).

Measurement of protein concentration. Protein concentration in crude extracts of bacteria was measured by the method of Lowry *et al.* (1951). A calibration curve was constructed by using bovine serum albumin as the standard. For rapid estimation of the protein concentration at later stages in the enzyme purification, the spectrophotometric method of Warburg & Christian (1941) was used. The A_{280} of a solution (1 mg/ml) of pure aldolase 2 in 10 mM-*N*-ethylmorpholine/acetic acid buffer, pH 7.5, was estimated to be 1.10 ± 0.02 by amino acid analysis of samples from a solution of measured absorbance.

Amino acid analysis. Dry salt-free samples (50 μ g) of aldolase 2 were hydrolysed in 0.25 ml of Aristar-grade 6M-HCl in sealed evacuated tubes at 105°C. 2-Mercaptoethanol (0.05%, v/v) was included in the hydrolysis acid to protect methionine and tyrosine (Keutmann & Potts, 1969; Houston, 1971). Triplicate samples were hydrolysed for each of 24, 48 and 72 h. The amino acids were analysed on a Rank-Hilger Chromaspek analyser coupled with a Digico micro 16V computer. Serine and threonine values were corrected for destruction on hydrolysis by extrapolation to zero time of hydrolysis. Methionine values were taken from the 24 h hydrolysates only, and valine and isoleucine values from the 72 h hydrolysates only. *S*-Carboxymethylcysteine values were taken from 24 h hydrolysates of *S*-carboxymethylated aldolase 2. Tryptophan determinations were made possible by hydrolysing the protein with 6M-HCl containing thioglycollic acid (2%, v/v) (Matsubara & Sasaki, 1969). To compare different analyses, the amount of each amino acid was expressed as a fraction of the total amount of asparagine, glutamic acid, glycine, alanine, tyrosine, phenylalanine, histidine and lysine in the analysis.

***S*-Carboxymethylation.** Aldolase 2 was reduced and *S*-carboxymethylated in 0.1M-Tris/HCl buffer, pH 8.5, in the presence of 8M-urea as described by Anderson *et al.* (1969).

Polyacrylamide-gel electrophoresis. Polyacrylamide-gel electrophoresis of aldolase 2, in the presence and absence of 8M-urea was performed by the method of Davis (1964) as described by Anderson *et al.* (1969). Aldolase 2 was also examined by electrophoresis in 7.5% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS (Shapiro & Maizel, 1969; Weber & Osborn, 1969). The subunit molecular weight was estimated by comparing its mobility with those of bovine serum albumin (mol.wt. 68000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (subunit mol.wt. 36000), and hen egg-white lysozyme (mol.wt. 14300). A linear plot for log

(molecular weight) against electrophoretic mobility was obtained.

N-Terminal analysis. *S*-Carboxymethylated aldolase 2 was examined for free *N*-terminal residues by the dansyl procedure in 8M-urea (Gray & Hartley, 1963). Dansyl-amino acids were separated and identified by chromatography on polyamide thin-layer sheets by using standard methods (Woods & Wang, 1967; Hartley, 1970; Gray, 1972).

Cross-linking. Dimethyl suberimide dihydrochloride was stored in a sealed vial over desiccant at -20°C . Immediately before use it was made up as a solution (20mg/ml) in 100mM-*N*-ethylmorpholine/acetic acid buffer, pH8.0, containing 147mM-NaOH. A one-tenth volume was added to the protein solution to give a final reagent concentration of 2mg/ml. Cross-linking was performed in 100mM-ethylmorpholine/acetic acid buffer, pH8.0, at 20°C with an aldolase 2 concentration of 1mg/ml. After 3h incubation, the reaction was stopped by the addition of 2 drops of 3M-sodium acetate (pH5.0)/ml of reaction mixture. The protein was freed from salt by dialysis against water, and samples were examined by SDS/polyacrylamide-gel electrophoresis.

Immunological procedures. An immunological study of aldolase 2 was carried out with the kind assistance of Dr. R. A. Harrison (Department of Biochemistry, Imperial College of Science and Technology, London, U.K.). Anti-(aldolase 2) serum was prepared from a white laboratory rat by the intramuscular injection at two sites of a sonicated mixture comprising 0.8ml of phosphate-buffered saline (0.8% NaCl/0.02% KCl/0.115% Na_2HPO_4 /0.02% KH_2PO_4 , pH7.2), 0.8ml of complete Freund's adjuvant and 0.05ml of aldolase 2 (2.5mg/ml). After 10 days, an intravenous injection was given comprising 0.05ml of aldolase 2 (2.5mg/ml) and 0.45ml of 5mM-sodium phosphate buffer, pH7.2. A second intramuscular injection was administered 25 days after the first, to increase the amounts of aldolase-specific antibodies. The final bleed, from the tail vein, was performed 1 week later. The blood was allowed to clot, and the clotted erythrocytes and fibrin were removed by centrifugation. The serum was then stored at -20°C .

Ouchterlony plates were constructed on glass blanks (6cm $\times$ 6cm) by using 1% (w/v) agarose in 100mM-Tris/HCl buffer, pH8.5. Antiserum was placed in the central well, and the test samples in the outer wells. After overnight incubation in a moist box, the plates were pressed between filter papers for 30min and then dried. The plates were washed with water for 1h, dried again and then stained in 0.5% (w/v) Coomassie Brilliant Blue in methanol/acetic acid/water (9:2:9, by vol.) for 20min. They were destained overnight in methanol/acetic acid/water (25:1:24, by vol.) and then dried.

Cell-free extracts of bacteria for immunodiffusion experiments were prepared by sonicating cell sus-

pensions (0.2mg/ml) in 0.1M-Tris/HCl buffer, pH 7.75, containing 1mM-2-mercaptoethanol and 0.1mM-phenylmethanesulphonyl fluoride for 5min in a water-cooled Rapidis sonicator. The temperature remained below 23°C . The crude sonicated extracts were clarified by centrifugation at 15000 rev./min in the Sorvall SS34 rotor for 20min at 2°C . Yeast cells were broken by homogenization with glass beads in a Braun (Melsungen, Germany) homogenizer.

Ultracentrifugation. Sedimentation - equilibrium studies of aldolase 2 were carried out at 20°C in a Beckman model E analytical ultracentrifuge equipped with Rayleigh interference optics. The procedures of Nureddin & Johnson (1977) were used, and were similar to those reported by Charlwood (1965). Before use, protein solutions (approx. 3mg/ml) were well dialysed at 4°C against 50mM-Tris/HCl buffer, pH7.5 (at 20°C), containing 100mM-NaCl. The dialysed solution (70 μ l) was introduced into one limb of a double-sector cell, and the buffer (160 μ l) was introduced into the other limb. During the acceleration of the rotor a capillary 3mm from the base of the channels allowed the buffer to layer on to the solution. The interference fringes in the region of the meniscus were then repeatedly photographed until the attainment of equilibrium. The fringe shift caused by backward diffusion of the protein provided an accurate measure of the equilibrium concentration at the meniscus. After attainment of equilibrium, a full-length interference photograph was taken, and molecular weights were calculated from the slopes of $\ln c$ -against- x^2 plots (where c = protein concentration and x = radial distance). The buffer density was estimated by pycnometry to be 1.00475g/ml at 20°C , and a partial specific volume of 0.73 for aldolase 2 was calculated from its amino acid composition (Cohn & Edsall, 1943).

Results

Purification of aldolase 2

A simplified version of the procedure reported by Stribling & Perham (1973) was adopted for the purification of aldolase 2. It was carried out at room temperature (20°C) except where otherwise stated. The source of enzyme was a culture of *E. coli* (Crookes' strain) grown with aeration at 30°C in MRE 162 medium with a glycerol feed by the Microbiological Research Establishment, Porton Down, Wilts., U.K. Bacterial cell paste (500g) was suspended in 1.25 litres of 20mM-sodium phosphate buffer, pH7.0, and then sonicated in 50ml portions, each for 4min, in a Mullard SL82 sonicator. During sonication the temperature of the suspension remained below 20°C . The extract was then centrifuged for 30min at 2°C and 13000g (r_{av} . 14.6cm). 1M-Tris was used to adjust the pH of the clear supernatant to 7.5 at 20°C ,

and the resultant protein precipitate was removed by centrifugation as above. The supernatant was diluted with water to the same electroconductivity as 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, and was then applied to a column (13.5 cm × 9 cm) of DEAE-cellulose equilibrated with this buffer. After loading, the column was washed with 3 litres of the same buffer, followed by a linear gradient (10 litres) of 50–250 mM-NaCl in 50 mM-Tris/HCl buffer, pH 7.5, at a flow rate of 2.4 litres/h. Fractions (40 ml) were collected and assayed for aldolase activity, protein concentration and electroconductivity. A single peak of EDTA-sensitive aldolase activity assayed in the presence and absence of 5 mM-EDTA was eluted at an NaCl concentration of approx. 150 mM. Fractions containing aldolase activity were pooled (total volume approx. 2.5 litres) and then diluted to an electroconductivity lower than that at which the aldolase activity was first eluted from the column. The solution was concentrated by applying it to a small column (11 cm × 6 cm) of DEAE-cellulose equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, and then eluting the enzyme in a small volume (approx. 600 ml) of buffer containing 250 mM-NaCl.

Further purification of the enzyme was achieved by the stepwise addition of solid $(\text{NH}_4)_2\text{SO}_4$, at 2 °C. Almost all of the aldolase was precipitated between 55 and 80% saturation of the solution with $(\text{NH}_4)_2\text{SO}_4$, and was collected by centrifugation for 20 min at 2 °C and 13 000 *g* (r_{av} , 14.6 cm). The precipitate was redissolved in a small volume of 75 mM-sodium phosphate buffer, pH 6.8, and dialysed at 4 °C against this buffer to remove the $(\text{NH}_4)_2\text{SO}_4$. The enzyme solution was then applied to a column (8 cm × 4 cm) of hydroxyapatite equilibrated with the same phosphate buffer. After the column had been washed with buffer until the protein concentration in the eluate had fallen to approx. 0.05 mg/ml, the aldolase was eluted in a small volume of a more concentrated (200 mM) sodium phosphate buffer, pH 6.8.

Protein was precipitated by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation and further fractionated by suspending the precipitate at 2 °C in a series of $(\text{NH}_4)_2\text{SO}_4$ solutions of gradually decreasing concentration. After each 'wash', the remaining precipitate was collected by centrifugation. The washes with highest specific activities were then pooled, and the protein was reprecipitated by the addition of $(\text{NH}_4)_2\text{SO}_4$. In the final step of the purification, the precipitate was dissolved in a small volume of 50 mM-Tris/HCl buffer, pH 7.5, and then subjected to gel filtration at 4 °C on a column (120 cm × 2 cm) of Sephadex G-150 equilibrated with this buffer. Fractions (4.4 ml) were collected and assayed for protein concentration and for aldolase activity. Those with the highest specific activities were pooled and then concentrated by ultrafiltration. The purified aldolase could be stored as a deep-frozen solution at -15 °C for many months without loss of activity. An overall yield of about 30% gave approx. 30 mg of aldolase 2 from 500 g of cell paste. The enzyme had a specific activity of approx. 27 i.u./mg when assayed at the pH optimum of 9 with the coupled assay. A summary of the purification is given in Table 1.

Characterization of aldolase 2

N-Terminal analysis. Serine was the only free *N*-terminal amino acid detected when a sample of *S*-carboxymethylated aldolase 2 was examined by the dansylation procedure.

Polyacrylamide-gel electrophoresis. Aldolase 2 gave a single major band of protein when examined by electrophoresis in the absence of denaturing agents. Similarly, *S*-carboxymethylated aldolase 2 ran as a single major band on polyacrylamide gels in the presence of 8 M-urea or of SDS (0.1%, w/v). These results, together with the occurrence of only one free *N*-terminal amino acid in aldolase 2, suggest that the enzyme contains only one type of subunit. The

Table 1. *Purification of aldolase 2*

Units of activity were measured in the colorimetric assay; for further details see the text. Protein concentration was measured by the procedure of Lowry *et al.* (1951)^a, by that of Warburg & Christian (1941)^b or from A_{280} ^c.

Procedure	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
1. Extraction	51 000	11 100 ^a	4.6	100	1
2. DEAE-cellulose chromatography	39 000	835 ^b	46.7	77	10
3. $(\text{NH}_4)_2\text{SO}_4$ (55–80% satd.) precipitation	36 500	366 ^c	99.7	72	22
4. Hydroxyapatite chromatography	38 500	115 ^c	333	75	72
5. $(\text{NH}_4)_2\text{SO}_4$ 'wash'	18 500	43 ^c	431	36	93
6. Sephadex G-150 gel filtration	15 300	32 ^c	477	30	104

mobility of the protein band on SDS/polyacrylamide gels corresponded with a subunit molecular weight of approx. 42000, a value distinctly greater than that of 34500 obtained for the enzyme previously isolated (Stribling & Perham, 1973).

Gel filtration. An estimate of the molecular weight of native aldolase 2 was obtained by comparing its elution volume from a column (120 cm $\times$ 2 cm) of Sephadex G-150 with the elution volumes for proteins of known molecular weight (Andrews, 1964). Horse heart cytochrome *c* (mol.wt. 11700), ovalbumin (mol.wt. 43000), bovine serum albumin (mol.wt. 68000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (mol.wt. 144000) and rabbit muscle aldolase (mol.wt. 160000) were used to calibrate the column. Elution was performed at 4°C with 50 mM-Tris/HCl buffer, pH 7.5. Aldolase 2 was eluted just before bovine serum albumin, and its molecular weight was estimated as approx. 80000. The single peak of EDTA-sensitive aldolase activity obtained by gel filtration of crude cell-free extracts of *E. coli* (Crookes' strain) was also eluted in this position. The elution volume was unaffected by the inclusion of either 2-mercaptoethanol or the serine-proteinase inhibitor phenylmethanesulphonyl fluoride in the buffers used during the preparation and chromatography of the extracts. This suggests that the purified aldolase 2 represents the only class-II fructose 1,6-bisphosphate aldolase in *E. coli*, and closely resembles the form found *in vivo*.

Ultracentrifugal analysis. The molecular weight of aldolase 2 was more accurately determined by sedimentation-equilibrium studies. These were carried out with protein solutions of approx. 3 mg/ml or approx. 1.8 mg/ml, and rotor speeds of 10000, 12000 and 14000 rev./min. In all cases, plots of $\ln c$ against x^2 were linear. From the slope of these plots the molecular weight of aldolase 2 was estimated to be 78000 ± 2000 .

Cross-linking of aldolase 2. The dimeric nature of aldolase 2 was supported by the results of cross-linking studies. After incubation with dimethyl suberimidate (2 mg/ml) for 3 h, aldolase 2 (1 mg/ml) comprised mainly a single species of higher molecular weight (Fig. 1). The molecular weight of this species on SDS/polyacrylamide gels was estimated to be 84000. No material of yet higher molecular weight was produced. In a parallel experiment, samples of aldolase 2 were incubated with 5 mM-EDTA for 45 min at 20°C before addition of the cross-linking reagent. Although this procedure completely abolished enzymic activity, it had no effect on the cross-linking pattern produced by incubation with dimethyl suberimidate for 3 h, suggesting that the bivalent metal ion does not play a structural role in the enzyme. The same is true of yeast aldolase (Harris *et al.*, 1969).

Amino acid analysis. The amino acid composition

Fig. 1. Electrophoretic analysis on SDS/polyacrylamide gels of aldolase 2 before (a) and after (b) treatment for 3 h at 20°C with dimethyl suberimidate (2 mg/ml)

of aldolase 2 given in Table 2 was calculated assuming a subunit mol.wt. of 40000. Analysis of the data by the least-squares procedure of Hoy *et al.* (1974) showed that this molecular weight gave the best fit to integral residue numbers, supporting the value obtained by SDS/polyacrylamide-gel electrophoresis. Table 2 also includes the amino acid compositions of the class-II aldolases from the yeast *Saccharomyces cerevisiae* (Harris *et al.*, 1969) and the thermophilic bacterium *Bacillus stearothermophilus* (Jack, 1973). To make an objective comparison, the composition divergence, D (Harris & Teller, 1973) was calculated for each of the three pairs of proteins (Table 3). The low D value for yeast aldolase and *E. coli* aldolase 2 is comparable with that for the homologous aldolases from rabbit muscle and liver (Harris *et al.*, 1969). This suggests that these class-II aldolases may be evolutionarily related, especially since they are the same size and have other features in common. The enzyme from another prokaryote, *B. stearothermophilus*, is less similar to aldolase 2 in size (mol.wt. 60600; Sugimoto & Nosoh, 1971), and in amino acid composition (Tables 2 and 3) than is the enzyme from the eukaryote yeast.

Table 2. *Amino acid composition of class-II aldolases*

The amino acid composition of aldolase 2 was determined as described in the text. Results are expressed as means $\pm$ S.E.M. for the numbers of values given in parentheses. For serine and threonine the S.E.M. quoted is that for six 24 h hydrolysates. Cysteine was quantified as *S*-carboxymethylcysteine. The subunit molecular weight of the *E. coli* enzyme was taken as 40000. Composition for the *S. cerevisiae* enzyme was taken from Harris *et al.* (1969); subunit mol.wt. 40000. Composition for the *B. stearothermophilus* enzyme was taken from Jack (1973); subunit mol.wt. 31000.

Amino acid	Composition (mol/mol of subunit)		
	Aldolase 2 of <i>E. coli</i>	Yeast (<i>Saccharomyces cerevisiae</i>)	<i>Bacillus stearothermophilus</i>
Lys	25.6 $\pm$ 0.3 (12)	25.3	17.2
His	12.1 $\pm$ 0.1 (12)	10.9	4.9
Arg	8.6 $\pm$ 0.3 (6)	9.5	9.1
Cys	4.2 $\pm$ 0.1 (6)	4.6	3.0
Asx	39.4 $\pm$ 0.3 (12)	39.4	20.7
Thr	18.0 $\pm$ 0.2 (6)	18.5	11.3
Ser	24.9 $\pm$ 0.4 (6)	19.3	13.8
Glx	36.7 $\pm$ 0.4 (12)	38.8	30.0
Pro	15.7 $\pm$ 0.4 (6)	16.3	11.1
Gly	31.9 $\pm$ 0.3 (12)	29.9	27.1
Ala	34.8 $\pm$ 0.3 (12)	33.5	24.9
Val	29.5 $\pm$ 0.3 (3)	22.3	19.7
Met	5.8 $\pm$ 0.1 (6)	6.9	7.0
Ile	21.0 $\pm$ 0.3 (3)	24.2	18.8
Leu	29.9 $\pm$ 0.6 (12)	22.6	16.0
Tyr	12.9 $\pm$ 0.2 (12)	12.1	5.1
Phe	12.9 $\pm$ 0.2 (12)	14.6	6.6
Trp	3.8 $\pm$ 0.2 (3)	4.6	Not determined

Table 3. *Amino acid-composition divergence matrix for class-II aldolases*

The values were calculated by the method of Harris & Teller (1973).

	D value		
	Aldolase 2	Yeast	<i>B. stearothermophilus</i>
Aldolase 2	0	0.033	0.056
Yeast	0.033	0	0.052
<i>B. stearothermophilus</i>	0.056	0.052	0

Immunological comparison of aldolases. Anti-(aldolase 2) serum reacted with purified aldolase 2 to give a single precipitin line in immunodiffusion experiments (Fig. 2). No reaction was observed with aldolase 1 from *E. coli*, with rabbit muscle aldolase, or with a semi-purified preparation of aldolase from *B. stearothermophilus* (kindly provided by Dr. D. McKay, M.R.C. Laboratory of Molecular Biology, Cambridge, U.K.). Immunodiffusion experiments were also performed with cell-free extracts prepared from various bacteria and yeast. The EDTA-sensitive aldolase activity of these extracts indicated that they contained between 0.15 and 0.6 mg of class-II aldolase/ml. Single precipitin lines were given by extracts of *E. coli* grown on glycerol (Fig. 2), on pyruvate and on lactate. However, no reaction was observed with extracts of *Lactobacillus casei*, *B. stearothermophilus* or *Saccharomyces cerevisiae*,

despite the use of a wide range of concentration of antiserum and of aldolase in each case.

Kinetic studies on aldolase 2

Variation of K_m and V_{max} with pH. Fig. 3 shows the effect of pH on the Fru-1,6- P_2 -cleavage activity of aldolase 2. The high pH optimum of 9 is unusual for a class-II enzyme (Rutter, 1964), but confirms the findings of Doelle & Manderson (1971), who used the colorimetric assay in their study of a class-II aldolase from *E. coli*. However, our K_m value for Fru-1,6- P_2 at pH 9.0, approx. 0.12 mM, is much lower than the value of 1.76 mM reported by these authors.

Effect of added K^+ ions. Neither KCl nor potassium acetate at concentrations of up to 200 mM enhanced the activity of aldolase 2 when it was assayed in glycylglycine buffer at any pH between 7.25 and 9.25.

Fig. 2. Immunodiffusion patterns obtained with aldolase 2

The central wells contained anti-(aldolase 2) serum, and the outer wells the test solutions. Wells 1, 2 and 3 respectively contained undiluted, 3-fold diluted and 9-fold diluted solutions of aldolase 2 (2.5 mg/ml), as did wells 4', 5' and 6'. Wells 4, 5 and 6 respectively contained undiluted, 3-fold diluted and 9-fold diluted samples from an extract of glycerol-grown *E. coli*. Wells 1', 2' and 3' respectively contained undiluted, 3-fold diluted and 9-fold diluted samples from an extract of the yeast *Saccharomyces cerevisiae*.

Fig. 3. Variation of the kinetic constants for aldolase 2 with pH

The Michaelis constant (●) and the maximum velocity (○) for fructose 1,6-bisphosphate cleavage were measured at 25°C in 80 mM-glycylglycine buffer adjusted to the appropriate pH with NaOH.

In fact, the higher concentrations were somewhat inhibitory. The picture was rather different when the assay buffer was 80 mM-Tris/HCl. The activity in this buffer at pH 7.5 was virtually the same as in glycylglycine at the same pH, and added K^+ ions had no effect. However, at pH 9.0 the activity was only 5%

Fig. 4. Effect of thiol compounds on the activity of aldolase 2

Aldolase 2 was assayed in the presence of various concentrations of 2-mercaptoethanol (●) and of cysteine (○) by using the coupled assay system in 80 mM-glycylglycine buffer, pH 9.0.

of that in glycylglycine buffer, and the addition of 40 mM-KCl stimulated the activity 1.4-fold. Further additions of KCl had no effect.

Effect of thiol compounds and bivalent metal ions. Fig. 4 shows the effect of cysteine and of 2-mercaptoethanol on the activity of aldolase 2 in the coupled assay at pH 9.0. Cysteine was by far the more inhibitory. It probably acted by chelating metal ions, since the inhibition was partially relieved by the addition of zinc salts. For example, the addition of 5 mM- $ZnSO_4$ to an assay containing 10 mM-cysteine increased the activity from 6 to 64% of that occurring in the absence of cysteine. In the absence of cysteine, Zn^{2+} salts had no effect. However, Zn^{2+} , Co^{2+} and Mn^{2+} ions (5 mM) substantially restored the activity of aldolase 2 that had been inactivated by the addition of 1 mM-EDTA to the assay.

Discussion

The typical class-II fructose 1,6-bisphosphate aldolase from the eukaryotic yeast *Saccharomyces cerevisiae* was first isolated in an unstable proteolytically degraded form with a mol.wt. of approx. 70000 (Richards & Rutter, 1961). After the adoption of precautions against proteolysis, a more stable form of mol.wt. approx. 80000 was isolated (Harris *et al.*, 1969). This pattern was repeated during the study of aldolase 2 from *E. coli*. It was first isolated in an unstable form having a mol.wt. of approx. 70000 (Stribling & Perham, 1973; Stribling, 1974). The isolation of another, probably undegraded, form of mol.wt. approx. 80000 has been described in the present paper.

Although the undegraded form of aldolase 2 loosely resembles the class-II aldolase from yeast in

size and amino acid composition, its kinetic properties are rather atypical. The pH optimum for Fru 1,6- P_2 cleavage is higher than that of most class-II enzymes (Rutter, 1964) and, unlike many of these aldolases (Morse & Horecker, 1968), aldolase 2 is relatively insensitive to compounds that react with thiol groups. It resembles the smaller aldolase from *B. stearothermophilus* in having no requirement for thiol compounds either for stability or activity, and in being unaffected by K^+ ions except when assayed in Tris buffers (Hill *et al.*, 1976). The characteristic effect of K^+ ions on the activity of most class-II aldolases is probably caused by an increased rate of carbanion formation (Riordan & Christen, 1969). It has been suggested that K^+ ions have no effect on *B. stearothermophilus* aldolase because carbanion formation is not normally the rate-limiting step for this enzyme. The expected stimulation of activity by K^+ ions is seen in Tris buffers probably because the latter interacts with the active-site transition-metal ion, causing carbanion formation to become rate-limiting (Hill *et al.*, 1976). Since the enzyme from the blue-green alga *Anacystis nidulans* (Willard & Gibbs, 1975) is similarly unaffected by K^+ ions, stimulation of activity by K^+ ions can no longer be regarded as a property of all class-II aldolases. Indeed, in view of the diversity of size and kinetic properties found in this class, perhaps the only truly characteristic feature of these enzymes is that they contain a catalytically essential bivalent metal ion.

Immunological techniques have been successfully used to investigate relationships among the aldolases of the lactic acid bacteria, showing that these class-II enzymes are homologous despite their widely differing sizes (London & Kline, 1973; London, 1974). Aldolase 2 from *E. coli* did not show any immunological identity with other class-II aldolases in the present study. This is perhaps not surprising in view of the great phylogenetic differences involved, and does not rule out a common origin for these enzymes. For example, the tissue-specific isoenzymes of mammalian aldolase are known to be homologous, and yet they show no immunological similarity to one another in immunodiffusion experiments (Penhoet *et al.*, 1969). However, the similarity in size and amino acid composition of *E. coli* aldolase 2 and yeast aldolase strongly suggests that they are related.

The relationships between class-II aldolases from such distantly related groups as the prokaryotic bacteria and blue-green algae, and the eukaryotic green algae and fungi, are likely to be established with certainty only by amino acid-sequence analysis. Although no sequence data for class-II aldolases have yet been published, the amino acid sequences around the cysteine residues of yeast aldolase have been investigated (Jack & Harris, 1971; Jack, 1973). The isolation of an undegraded form of *E. coli* aldolase 2 described in the present paper has enabled

similar studies to be carried out on this prokaryote enzyme. A comparison of amino acid sequence data for the two enzymes (S. A. Baldwin, J. I. Harris, R. S. Jack and R. N. Perham, unpublished work) confirms and extends the results reported here.

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