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Novel Kinetic and Structural Properties of the Class-I D-Fructose 1,6-Bisphosphate Aldolase from *Escherichia coli* (Crookes' Strain)

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Investigation of aldolase 1, the class-I D-fructose 1,6-bisphosphate aldolase (EC 4.1.2.13) from *Escherichia coli* (Crookes' strain), showed it to have unusual kinetic and structural properties. The enzyme appeared to be larger than was previously supposed and may be a decamer with a mol. wt. of approx. 340000. Its fructose 1,6-bisphosphate-cleavage activity was greatly enhanced by citrate, phosphoenolypyruvate, 2-oxoglutarate and *sn*-glycerol 3-phosphate. In contrast, its fructose 1-phosphate-cleavage activity was unaffected by these compounds. The enhancement exhibited a strong dependence on pH. These novel kinetic properties do not seem to be shared by any other fructose 1,6-bisphosphate aldolase, but recall the activation by polycarboxylic acids of the deoxyribose 3-phosphate aldolases from some other organisms. In view of its unusual properties, it is unlikely that aldolase 1 from *E. coli* is closely related to the class-1 aldolases that have been detected in several other prokaryotes, or to the typical class-I enzymes from eukaryotes.

Class-I fructose 1,6-bisphosphate aldolases were once thought to be confined to eukaryotic organisms (see review by Rutter, 1964), but have since been detected in several bacterial species. These newly discovered enzymes from prokaryotes form a rather diverse group of proteins, and it is not yet known whether any of them is related to the family of homologous class-I aldolases from eukaryotes. In some species, e.g. *Peptococcus aerogenes* (Lebherz & Rutter, 1973), they comprise the sole fructose 1,6-bisphosphate aldolase activity, whereas in others, e.g. *Escherichia coli* (Stribling & Perham, 1973) and *Lactobacillus casei* (London, 1974), both classes of fructose 1,6-bisphosphate aldolase are present. The latter organisms provide interesting systems in which the structural and functional relationships of the two types of enzyme can be investigated without the added complications of interspecies comparisons.

Aldolase 1 from *E. coli* was the first oligomeric class-I aldolase from a prokaryote to be characterized (Stribling & Perham, 1973). It was found to occur, together with a typical bacterial class-II enzyme (aldolase 2), in cultures grown on lactate or pyruvate, but not on glucose, which suggested that it might be preferentially involved in gluconeogenesis in this organism (Stribling & Perham, 1973). In many respects aldolase 1 appeared to resemble the typical class-I enzymes of eukaryotes. Thus it was oligomeric, possibly a tetramer, and showed an apparent mol.wt. of approx. 140000. Its activity was unaffected by EDTA, but it was inhibited by borohydride reduction in the presence of Fru-1,6-P_2 or dihydroxyacetone phosphate. Like mammalian aldolases, it cleaved fructose 1-phosphate, albeit slowly, and had a low K_m for Fru-1,6-P_2. However, the Lineweaver–Burk plot with Fru-1,6-P_2 was non-linear, and the possibility of proteolysis during the preparation of the enzyme was recognized (Stribling & Perham, 1973). The present paper describes a further investigation of this enzyme that has led to the discovery of some unusual kinetic and structural properties.

Abbreviations used: dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Fru-1,6-P_2, fructose 1,6-bisphosphate; SDS, sodium dodecyl sulphate.

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Materials and Methods

Materials

Aldolase 1 was prepared from lactate-grown *E. coli* (Crookes' strain) by the method of Stribling & Perham (1973) as modified in the present paper. Pig heart malate dehydrogenase, ox liver glutamate dehydrogenase, *E. coli* β-galactosidase, fructose 1,6-bisphosphate (trisodium salt), and fructose 1-phosphate (cyclohexylammonium salt) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Fructose 1,6-bisphosphate (tetrasodium salt), fructose 6-phosphate (disodium salt) and Zerolite 225 ion-exchange resin were the products of BDH Chemicals Ltd., Poole, Dorset, U.K. D,L-α-Glycerol phosphate (disodium salt) and dihydroxyacetone phosphate (cyclohexylammonium salt, dimethyl ketal) were purchased from Sigma Chemical Co.,

St. Louis, MO, U.S.A. 2-Oxoglutaric acid and protamine sulphate (from salmon testis) were bought from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Thrice-crystallized trypsin and pepsin were from the Worthington Biochemical Corp., Freehold, NJ, U.S.A. Phosphoenolpyruvate (cyclohexylammonium salt) was kindly given by Dr. H. B. F. Dixon of this Department. Sepharose 6B was from Pharmacia, Uppsala, Sweden, and silica-gel t.l.c. sheets (Polygram Sil G), the product of Machery, Nagel and Co., Düren, Germany, were obtained from Camlab, Cambridge, U.K. Bis(imidoesters) were synthesized and kindly provided by Dr J. R. Coggins, Department of Biochemistry, University of Glasgow. The provenance of all other enzymes and reagents has been described previously (Baldwin *et al.*, 1978). Analytical-grade chemicals and glass-distilled water were used throughout.

Methods

Enzyme assays. Except where otherwise stated, the coupled spectrophotometric procedure (Blostein & Rutter, 1963) was used to assay aldolase 1. It was performed as described by Baldwin *et al.* (1978), but with 80mM-Tris/HCl, pH7.5 (at 30°C), as the assay buffer, and with 2mM-citrate usually included as an activator. Purified Fru-1,6-P_2 was generally used as substrate. On one occasion the colorimetric assay for aldolase (Stribling & Perham, 1973) was used.

Purification of fructose 1,6-*bisphosphate.* The chromatographic procedure of Webster *et al.* (1976) was used for the purification of Fru-1,6-P_2. A sample (1g) of the commercial trisodium salt was dissolved in water and adjusted to pH2.65 with formic acid. The solution was diluted to the electroconductivity of 0.1M-pyridine/1M-formic acid buffer, pH2.65, and was then applied to a column (58cm×3cm) of DEAE-cellulose equilibrated with this buffer. Elution of the column with the same buffer produced two peaks of carbohydrate material, detected by staining after spotting-out on to paper (Trevelyan *et al.*, 1950). The second and major peak, which was eluted after 4 column volumes, contained Fru-1,6-P_2 and the solution was concentrated by rotary evaporation at 34°C. Water was added and the rotary evaporation repeated until the smell of pyridine was no longer detectable. The solution was then passed through a column (7cm×2cm) or Zerolit 225 resin (H^+ form) to remove pyridine, and was finally adjusted to pH6.0 with NaOH. The purified Fru-1,6-P_2 gave a single spot when stained for carbohydrate after paper electrophoresis at pH3.5. It was stored as a deep-frozen solution at −15°C.

Cross-linking. Cross-linking with dimethyl suberimidate was performed as described by Baldwin *et al.* (1978). Dimethyl glutarimidate dihydrochloride was used in the same way as the suberimidate, except that the stock solution was made up at 16.9mg/ml instead of 20mg/ml. Cross-linked samples were examined by electrophoresis on 5% (w/v) polyacrylamide gels containing SDS (0.1%, w/v).

Immunological procedures. The preparation of anti-(aldolase 1) serum, and the procedures used for immunodiffusion experiments, were similar to those used in the study of aldolase 2 from *E. coli* (Baldwin *et al.*, 1977). *Lactobacillus casei* was grown in M.R.S. Broth (Oxoid Ltd., London E.C.4, U.K.) at 30°C. A sample (10ml) from a 4h culture was used to inoculate each of five flasks containing 400ml of the growth medium. After 72h the cells were harvested by centrifugation for 30min at 13000*g* ($r_{av.}$ 14.6cm). They were washed in 100mM-Tris/HCl buffer, pH7.5, and stored at −20°C before use.

Ultracentrifugation. Sedimentation-equilibrium experiments were performed at 20°C in 50mM-Tris/HCl buffer, pH7.5, containing 100mM-NaCl, by procedures similar to those used in a study of aldolase 2 from *E. coli* (Baldwin *et al.*, 1977). A partial specific volume of 0.73 for aldolase 1 was calculated from its amino acid composition (Cohn & Edsall, 1943).

Peptide 'mapping'. *S*-Carboxy[^{14}C]methylated proteins (2mg/ml), suspended in NH_4HCO_3 solution (5g/l), were digested at 37°C for 4h with trypsin (1%, w/w). Peptic digests were performed in a similar fashion except that proteins were digested in formic acid (5%, v/v). Peptides were recovered by freeze-drying, and 50μg samples were taken for 'mapping'. This was carried out by the micro-method of Bates *et al.* (1975). The staining and radioautography of such 'maps' has been described (Bates *et al.*, 1975).

Miscellaneous techniques. Techniques not described above, such as the determination of protein concentration, amino acid analysis, *N*-terminal analysis, *S*-carboxymethylation and polyacrylamide-gel electrophoresis, were identical with those previously adopted for the study of aldolase 2 from *E. coli* (Baldwin *et al.*, 1978).

Results

Discovery of the novel kinetic properties of aldolase 1

The discovery of the novel kinetic properties of aldolase 1 stemmed from the use of the coupled assay for aldolase during attempts to purify the enzyme. Previously the colorimetric assay had been used (Stribling & Perham, 1973). The bacterial extracts used as the source of aldolase 1 contained a potent fructose 6-phosphate reductase activity that was probably due to D-mannitol 1-phosphate dehydrogenase, an NAD^+-specific enzyme known to occur in *E. coli* (Wolff & Kaplan, 1956). This enzyme manifested itself in the aldolase-coupled assay because of

the contamination by fructose 6-phosphate of all the commercial samples of Fru-1,6-P_2 tested. It was at first mistaken for aldolase 1 activity because not only did it greatly exceed the latter, it was also eluted in the same position as aldolase 1 when bacterial extracts were chromatographed on DEAE-cellulose. The true aldolase 1 activity could be measured by using Fru-1,6-P_2 that had been purified by chromatography on DEAE-cellulose to remove the fructose 6-phosphate. The problem of substrate contamination could also be circumvented by gel-filtering bacterial extracts on Sephadex G-150 to separate the two enzyme activities before assay. However, neither method revealed as much aldolase 1 activity in the extracts as had been expected from previous results (Stribling & Perham, 1973; Stribling, 1974). The measured activity was found to depend on the batch of commercial Fru-1,6-P_2 used in the assays, varying from one to four times that obtained with purified substrate, and a slow increase in enzyme rate during the assays was observed. This increase in rate was tracked down to activation of the enzyme by the end product of the assay, *sn*-glycerol 3-phosphate. Citrate, phosphoenolpyruvate and 2-oxoglutarate were also potent activators, stimulating the activity at least 20-fold when present at concentrations around 10mM. The enhanced activities seen in the coupled assay were dependent on the presence of coupling enzymes and Fru-1,6-P_2, they were not affected by 5mM-EDTA, and were observed both in Tris/HCl and in *N*-ethylmorpholine/acetic acid buffers. They were also seen in the colorimetric assay, confirming that they corresponded to a true aldolase activity. NaCl, phosphate, pyruvate, malate and acetate had no effect. Neither did the likely contaminants of Fru-1,6-P_2, namely fructose 6-phosphate and fructose 1-phosphate. None of the compounds tested had any marked effect on rabbit muscle aldolase or on aldolase 2 from *E. coli* when these enzymes were assayed under the same conditions as aldolase 1.

All the samples of Fru-1,6-P_2, which yielded different activities with aldolase 1 alone, yielded the same enhanced activity when an excess of activator was included in the assay (e.g. 20mM-citrate, pH7.5). This suggested that they might themselves be contaminated to different extents with some activator. Its identity remained unknown, although enzymic assay (Michal & Lang, 1974) showed that it was not *sn*-glycerol 3-phosphate. Presumably the high aldolase 1 activity previously detected in extracts of *E. coli* (Stribling & Perham, 1973) resulted from the use of a particularly 'active' batch of Fru-1,6-P_2 in the assays. After the problems associated with the coupled assay of aldolase 1 had become clear, purified Fru-1,6-P_2 was used for all assays and 2mM-citrate was included to increase their sensitivity. It then became possible readily to purify the enzyme, as we describe below.

Purification of aldolase 1

A proteinase inhibitor, phenylmethanesulphonyl fluoride (0.1mM) was included in buffers during the present purification of aldolase 1, since proteolysis was suspected to be a possible cause of non-linear Lineweaver–Burk plots obtained with this enzyme (Stribling & Perham, 1973). The source of aldolase was a protamine sulphate supernatant prepared from 1kg of lactate-grown *E. coli* (Crookes' strain) in the manner described by Stribling & Perham (1973). However, the subsequent purification procedure differed in the following respects from that used previously. In the first step, chromatography on a column (14cm×9cm) of DEAE-cellulose, a linear gradient (10 litres) of 0–100mM-NaCl in 50mM-Tris/HCl, pH7.5, was used because the enzyme was eluted at a higher NaCl concentration (approx. 55mM) than in previous preparations. A second chromatography step was then introduced, in which a linear gradient (5 litres) of NaCl in 50mM-Tris/HCl, pH7.5, was used to elute the enzyme from a smaller column (9cm×6.5cm) of DEAE-cellulose. The pooled fractions containing aldolase 1 were concentrated by ultrafiltration before the $(NH_4)_2SO_4$ step. Crystallization of the enzyme was not achieved, and so an $(NH_4)_2SO_4$ 'washing' procedure was used for further purification of the aldolase. In this process, the protein precipitate formed in 65%-satd. $(NH_4)_2SO_4$ was 'washed' with $(NH_4)_2SO_4$ solutions of successively lower concentrations, as described for the purification of aldolase 2 (Stribling & Perham, 1973; Baldwin *et al.*, 1978). This step brought the enzyme almost to homogeneity, but gel filtration in 50mM-Tris/HCl, pH7.5, on a column (120cm×2cm) of Sephadex G-150 at 4°C was necessary to remove a minor contaminant. The aldolase activity was eluted as a sharp peak coincident with A_{280}.

The purified enzyme had a specific activity of approx. 17i.u./mg when assayed at pH7.5 in the coupled assay containing 2mM-citrate. Its activity, measured in the presence or absence of activators, was completely destroyed by incubation with KBH_4 and dihydroxyacetone phosphate. The presence of activators was not necessary for this inactivation. SDS/polyacrylamide-gel electrophoresis gave a single band of protein with a mobility similar to that of rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, as expected (Stribling & Perham, 1973). In addition, examination of a sample of *S*-carboxymethylated enzyme by the dansyl procedure revealed only dansylthreonine, N^6-dansyl-lysine and *O*-dansyltyrosine, as found by Stribling (1974). An overall yield of about 30% gave approx. 40mg of aldolase 1 from 1kg of cell paste. The enzyme was stored as a deep-frozen solution at −15°C for several months without loss of activity. Table 1 summarizes the purification procedure.

Table 1. *Purification of aldolase 1 from E. coli*
The activity in the coupled assay was measured in the presence of 2mM-citrate. For further details, see the text.

Procedure	Total activity (i.u.)	Total protein (mg)	Specific activity (i.u./mg)	Yield (%)	Purification (fold)
1. Protamine sulphate supernatant	2400	15300*	0.16	100	1
2. DEAE-cellulose chromatography	1444	1614†	0.89	60	6
3. DEAE-cellulose chromatography	1125	447‡	2.5	47	16
4. $(NH_4)_2SO_4$ (50–65% satd.) precipitation	1035	182‡	5.7	43	36
5. $(NH_4)_2SO_4$ 'wash'	646	44‡	14.7	27	92
6. Sephadex G-150 gel filtration	675	40‡	16.9	28	106

* Protein concentration measured by the procedure of Lowry *et al.* (1951).
† Protein concentration measured by the method of Warburg & Christian (1941).
‡ Protein concentration measured from A_{280}.

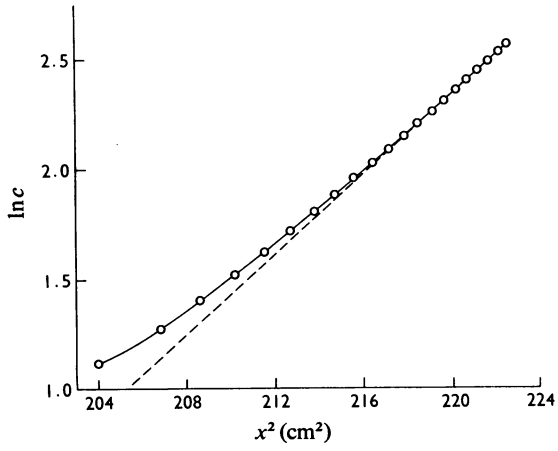


Fig. 1. *Determination of the molecular weight of aldolase 1 by sedimentation-equilibrium centrifugation*
The data are from a run performed at 20±0.1°C with an operating speed of 4400±5 rev./min in 50mM-Tris/HCl buffer, pH7.5, containing 100mM-NaCl, 1mM-EDTA and 1mM-2-mercaptoethanol. *c*, Protein concentration expressed in fringe numbers; *x*, radial distance (cm).

Molecular weight of aldolase 1

Gel filtration. When first isolated, the behaviour of aldolase 1 during gel filtration suggested that its molecular weight was approx. 140000 (Stribling & Perham, 1973). However, its elution volume when gel-filtered on Sephadex G-150 in the final step of the current purification suggests a much higher molecular weight. It was gel-filtered at 4°C together with proteins of known molecular weight on a column (57cm×0.65cm) of Sepharose 6B, by using 50mM-Tris/HCl buffer, pH7.5, containing 1mM-EDTA and 1mM-2-mercaptoethanol. A reasonably linear relationship between log(molecular weight) and elution volume was obtained for the marker proteins. Aldolase 1 was eluted in exactly the same position as ox liver glutamate dehydrogenase, suggesting that its molecular weight is approx. 320000.

Ultracentrifugal analysis. The size of aldolase 1 was further investigated by means of sedimentation-equilibrium studies. These were carried out at 20°C by using several different rotor speeds and protein concentrations. In all cases, plots of $\ln c$ (c = protein concentration) against x^2 (x = radial distance), were non-linear (Fig. 1), indicating some dissociation of the protein at low concentration. The molecular weight of aldolase 1, calculated from the slope of the linear region of the plots, was estimated to be 340000±20000. Addition of 1mM-EDTA and 1mM-2-mercaptoethanol to the buffers had no effect either on this value or on the general shape of the plots. Whatever the significance of the apparent dissociation of the protein in the ultracentrifuge, the enzyme lost no activity during these experiments.

Chemical cross-linking. In view of the unusually high molecular weight of aldolase 1 suggested by gel-filtration and ultracentrifuge studies, cross-linking was carried out to compare the structure with that of the well-characterized tetrameric aldolase from rabbit muscle. The influence of the activator of aldolase 1, citrate, on the cross-linking pattern was also examined in the hope of clarifying its mode of action. SDS/polyacrylamide gels of aldolase 1 and rabbit muscle aldolase, cross-linked by treatment with dimethyl suberimidate, are shown in Fig. 2. The rabbit enzyme yielded the expected pattern of cross-linked products, comprising mainly cross-linked tetramers after 4h incubation with cross-linker. However, the electrophoretic pattern of aldolase 1

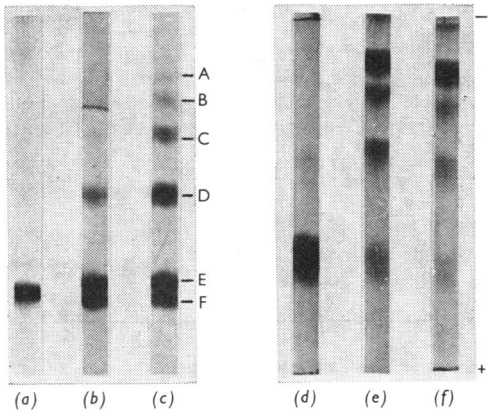


Fig. 2. *Cross-linking of aldolase* 1
SDS/polyacrylamide gels (5%, w/v) of aldolase 1 (*a*, *b*, *c*) and of rabbit muscle aldolase (*d*, *e*, *f*). Samples (*a*) and (*d*) were taken before cross-linking. Samples (*b*) and (*e*) were cross-linked at 23°C for 3h with dimethyl suberimidate (2mg/ml) in the presence of 10mM-citrate. Samples (*c*) and (*f*) were treated the same as (*b*) and (*e*), but in the absence of citrate.

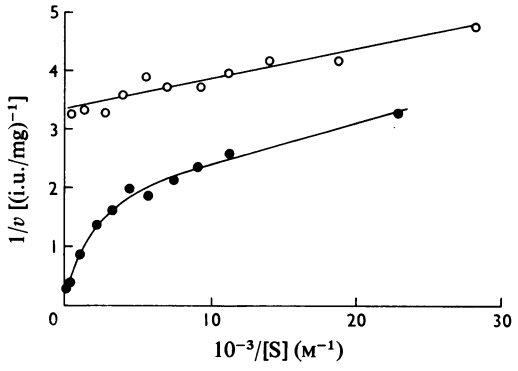


Fig. 3. *Lineweaver–Burk plots for aldolase* 1 *with different batches of fructose* 1,6-*bisphosphate*
One plot was obtained with a commercial sample of substrate (●), the other (○) by using the same batch of substrate after its repurification by chromatography on DEAE-cellulose. All assays were performed at pH7.5.

was very different, and showed several unusual features. Cross-linking was nowhere near as complete as for the rabbit enzyme, and each gel 'band' comprised several 'sub-bands'. This was particularly obvious at the monomer position, which was occupied by two well-resolved bands (E and F, Fig. 2). Hucho *et al.* (1975) observed a similar phenomenon during cross-linking of catalase, and attributed the anomalous mobilities to incomplete unfolding of the polypeptide in SDS after the formation of intrachain cross-links. Comparison of the mobilities of the major bands A, B, C, D and E/F (Fig. 2) in cross-linked aldolase 1 with those of components from the pyruvate dehydrogenase multienzyme complex of *E. coli* (Perham & Thomas, 1971) confirmed that the bands comprised pentameric, tetrameric, trimeric, dimeric and monomeric products respectively. The same pattern was obtained by using protein concentrations of either 0.2 or 1 mg/ml during cross-linking, and so it is unlikely that the pentamer band resulted from intermolecular cross-linking.

SDS/polyacrylamide-gels of untreated aldolase 1 also occasionally showed some dimeric material, but densitometry revealed that the amount was very small. It may have resulted from disulphide cross-linking, although gel samples were heated to 100°C in the presence of 2-mercaptoethanol before loading. Densitometry of the gels confirmed that much more dimer was present in the dimethyl suberimidate-cross-linked samples, except when 10mM-citrate was present during the cross-linking. In this case the amount of dimer present was severely decreased, and very little higher-molecular-weight material was present (Fig. 2). However, citrate also seemed to decrease the cross-linking of rabbit muscle aldolase, although to a lesser extent (Fig. 2). To try to increase the cross-linking of aldolase 1, the effect of the shorter-chain-length reagent, dimethyl glutarimidate, on both this enzyme and rabbit muscle aldolase was examined. No gel bands corresponding to species larger than dimers were found in either case and the extent even of dimer formation was very small. Interestingly, a doublet monomer band was not obtained for aldolase 1 with this reagent.

Kinetic behaviour of aldolase 1

Further investigations of the unusual kinetic behaviour of aldolase 1 were carried out on the purified enzyme. The effect of substrate concentration on the reaction rate was found to depend on the type of Fru-1,6-P_2 used for the assays. Commercial Fru-1,6-P_2 yielded a non-linear Lineweaver–Burk plot (Fig. 3) that resembled that previously reported for aldolase 1 (Stribling & Perham, 1973). In contrast, the plot obtained when purified Fru-1,6-P_2 was used was linear over the same concentration range (Fig. 3). This suggests that an activator contaminating commercial preparations of Fru-1,6-P_2 is responsible for the non-linear kinetics, rather than partial degradation of the enzyme itself. However, even the purified substrate gave a non-linear plot when this was extended to lower substrate concentrations (Fig. 4). This may once more have been due to a contaminant

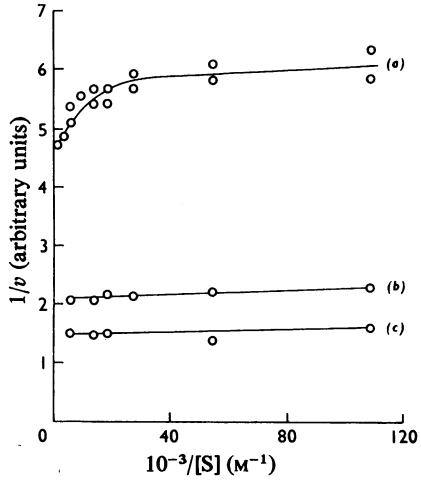


Fig. 4. *Effect of citrate on Lineweaver–Burk plots for aldolase* 1 *with fructose* 1,6-*bisphosphate*
All assays were performed at pH7.5 with repurified fructose 1,6-bisphosphate. Plot (*a*) was obtained in the absence of citrate, plot (*b*) in the presence of 50 μM-citrate and plot (*c*) in the presence of 100 μM-citrate.

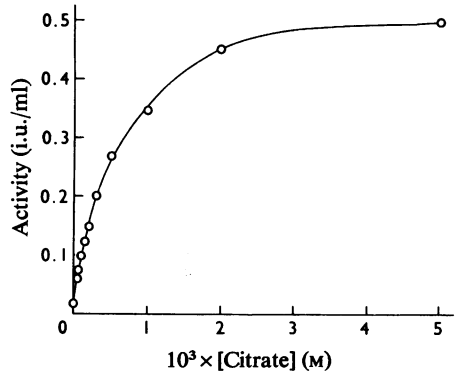
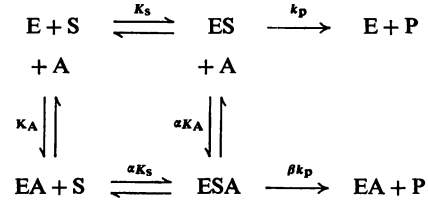


Fig. 5. *Effect of citrate on the activity of aldolase* 1
Assays were performed at pH7.5 using a fixed concentration (3.7mM) of repurified fructose 1,6-bisphosphate.



Scheme 1.
Key to symbols: E, enzyme; S, substrate; A, activator; P, product; K_s and K_A are dissociation constants; k_p is a rate constant; α and β are numerical factors.

remaining in the substrate, or may have been caused by some other artifact of the assay.

The product activation inherent in the method of assay renders it unsatisfactory for accurate kinetic studies. However, it was used for a further examination of the effect of citrate and phosphoenolpyruvate on the enzyme. Surprisingly, these compounds had no effect on the fructose 1-phosphate-cleavage activity of aldolase 1. When assayed at a fructose 1-phosphate concentration of 2mM this was about 60% of the non-activated Fru-1,6-P_2-cleavage activity (measured at an Fru-1,6-P_2 concentration of 3.7mM). Fig. 4 illustrates the influence of citrate on Lineweaver–Burk plots of aldolase 1 with Fru-1,6-P_2. Although an effect on the K_m for Fru-1,6-P_2 is not precluded, it is obvious that the major effect of citrate is to increase the $V_{max.}$ for Fru-1,6-P_2 cleavage. In view of this observation, and of the non-linearity of the Lineweaver–Burk plots, all subsequent experiments were conducted at a fixed concentration (3.7mM) of Fru-1,6-P_2. The measured activities were assumed to differ from apparent $V_{max.}$ values by an insignificant amount. Fig. 5 demonstrates the activation of the enzyme by increasing concentrations of citrate. The simple model (Segel, 1975) shown in Scheme 1 was used in the further analysis of this effect.

If it be assumed that all the equilibria shown in Scheme 1 are rapid compared with the rate of breakdown of the ES or ESA complexes to form products, then it can be shown that the following equation applies at any activator concentration:

$$V'_{max.} = \frac{V_{max.}(1+\beta[A])/\alpha K_A}{(1+[A])/\alpha K_A}$$

where $V'_{max.}$ is the apparent maximum velocity at any activator concentration, and $V_{max.}$ is that found in the absence of activator. It follows that:

$$\frac{1}{V'_{max.}-V_{max.}} = \frac{\alpha K_A}{(\beta-1)V_{max.}} \cdot \frac{1}{[A]} + \frac{1}{(\beta-1)V_{max.}}$$

Consequently, a graph of $1/(V'_{max.}-V_{max.})$ versus $1/[A]$ is a straight line from whose gradient and intercept the values of β and αK_A can be determined.

When the data in Fig. 5 were treated in this fashion, making the assumption that they corresponded to apparent $V_{max.}$ values, a linear graph was obtained (Fig. 6). From its slope and intercept, values for β (30.4) and αK_A (0.53mM) were calculated. Separate values for α and K_A were not determined because the variation with citrate concentration of the K_m for

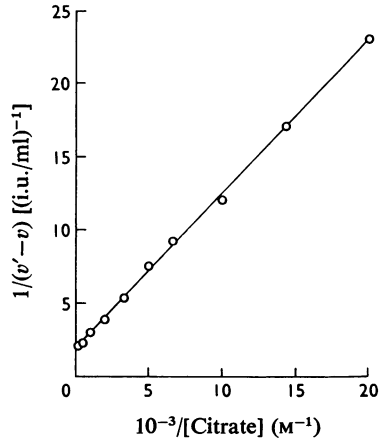


Fig. 6. *Effect of citrate on the activity of aldolase* 1
The data from Fig. 5 have been replotted in double-reciprocal form. v', Activity in the presence of citrate: v, activity in its absence.

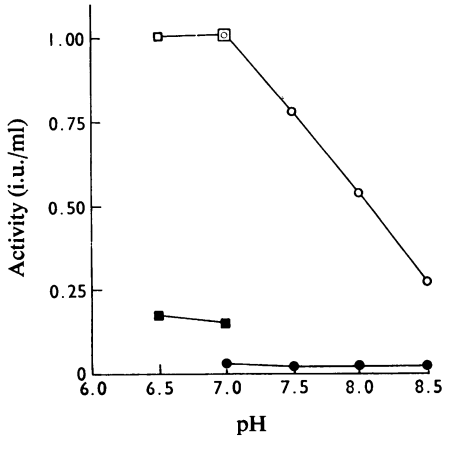


Fig. 7. *Effect of pH on the activity of aldolase* 1
The activity of aldolase 1 was measured at a fixed concentration (3.7mM) of fructose 1,6-bisphosphate in the presence (○, □) and absence (●, ■) of 1mM-citrate. Tris/HCl (○, ●) and imidazole/HCl (□, ■) buffers (80mM) were used in the assays.

Fru-1,6-P_2 could not be measured. Although it is unlikely that the measured αK_A value represents the true dissociation constant envisaged in the simple model for activation, αK_A is a very useful parameter corresponding to the activator concentration that gives half-maximal activation. Similarly, β represents the maximum activation produced by an infinite concentration of activator. These two parameters were valuable in quantifying the effect of pH on the activation of aldolase 1. The influence of pH on the activity of aldolase 1 measured in the presence and absence of a fixed concentration of citrate (1mM) is shown in Fig. 7. The activity measured in the absence of activator was almost constant between pH7 and 8.5, whereas in the presence of citrate it rapidly increased with decreasing pH. Maximum activity was obtained at pH7. However, there was an added complication in that the imidazole buffer used for the lower pH assays itself appeared to have a small activating effect. To clarify the effect of pH, β and αK_A values were determined for phosphoenolpyruvate and citrate at two pH values, 7.5 and 8.0 (Table 2).

From these results it is obvious that the major effect of pH is to change the value of αK_A. Since both activators have pK values in roughly the same region as the pH values at which they are maximally effective, it is possible that the protonated forms of these compounds represent the true activators. However, a more detailed study will be required to decide whether ionization of a group on the enzyme or on the activator, or both, is responsible for the effect of pH.

Structural comparisons between aldolase 1 *and other aldolases*

Amino acid composition. The amino acid composition of our preparation of aldolase 1 is given in Table 3. It differs little from that previously reported (Stribling & Perham, 1973), except in the values for a few of the amino acids. The present values are more likely to be correct, since the analyses were more exhaustive than previously. In particular, 2-mercaptoethanol (0.5ml/litre) was included in the hydrolysis acid to increase the yield of *S*-carboxymethylcysteine. Table 3 also includes the compositions of aldolase 2 from *E. coli* (Baldwin *et al.*, 1977), of rabbit muscle aldolase, and of the class-I aldolase from *Peptococcus aerogenes*. Comparison of these compositions by the method of Harris & Teller (1973) revealed no very low values of composition divergence (Table 4), suggesting that aldolase 1 is not related to any of these enzymes.

Peptide 'mapping'. Comparison of *S*-carboxy-[^{14}C]methylated aldolases 1 and 2 from *E. coli* by peptide 'mapping', after tryptic or peptic digestion, revealed no striking similarities, either in the pattern of unlabelled or of radioactive peptides. A general similarity of the 'maps' probably resulted more from the techniques used to prepare them than from sequence homology between the proteins.

Immunological comparisons. The reaction of anti-(aldolase 1) serum with cell-free extracts of a variety of organisms was investigated. Before use, these extracts were assayed to determine their aldolase

Table 2. *Values of β and αK_A for the activation of aldolase* 1 *by phosphoenolpyruvate and citrate*
For details, see the text.

	β		αK_A	
pH	Phosphoenol-pyruvate	Citrate	Phosphoenol-pyruvate	Citrate
7.5	42.9	30.4	0.20	0.53
8.0	41.4	35.6	0.38	1.31

Table 3. *Amino acid composition of class-I and -II aldolases*
The amino acid composition of aldolase 1 was determined as described in the text. Results are expressed as means ±S.E.M. with the number of values used to calculate the mean given in parentheses. For serine and threonine the S.E.M. is quoted for the mean of three 24h hydrolysates. Cysteine was quantified as *S*-carboxymethylcysteine. The subunit mol.wt. was taken as 34500. *Peptococcus aerogenes* aldolase has a subunit mol.wt. of 33000 (Lebherz *et al.*, 1973). Rabbit muscle aldolase (A) has a subunit mol.wt. of 40000 (Anderson *et al.*, 1969). Aldolase 2 from *E. coli* has a subunit mol.wt. of 40000 (Baldwin *et al.*, 1978).

	Amino acid composition (mol/mol of subunit)			
Amino acid	Aldolase 1 of *E. Coli*	Class-I aldolase of *P. aerogenes*	Class-I aldolase of rabbit muscle	Aldolase 2 of *E. coli*
Lys	13.4±0.2 (9)	25	26.6	25.6
His	7.7±0.1 (9)	4	10.1	12.1
Arg	17.9±0.7 (9)	11	14.9	8.6
Cys	4.5±0.04 (9)	1	7.5	4.2
Asx	39.1±0.2 (9)	36	28.7	39.4
Thr	17.6±0.2 (3)	10	21.4	18.0
Ser	19.2±0.4 (3)	17	20.3	24.9
Glx	21.0±0.3 (9)	49	40.9	36.7
Pro	5.0±0.1 (9)	6	18.6	15.7
Gly	28.5±0.2 (9)	14	29.6	31.9
Ala	40.6±0.3 (9)	32	40.5	34.8
Val	21.9±0.1 (3)	15	21.1	29.5
Met	7.5±0.2 (9)	11	3.3	5.8
Ile	17.8±0.7 (2)	21	19.5	21.0
Leu	30.1±0.7 (12)	29	33.0	29.9
Tyr	18.4±0.4 (8)	8	10.9	12.9
Phe	6.9±0.2 (7)	11	7.7	12.9
Trp	Not determined	2	3.0	3.8

Table 4. *Amino acid-composition divergence matrix for aldolases*

	E. coli aldolase 2	*E. coli* aldolase 1	*Peptococcus aerogenes* aldolase	Rabbit muscle aldolase
E. coli aldolase 2	0	0.080	0.096	0.054
E. coli aldolase 1	0.080	0	0.128	0.090
P. aeorgenes aldolase	0.096	0.128	0	0.094
Rabbit muscle aldolase	0.054	0.090	0.094	0

content, by using purified Fru-1,6-P_2 and KCl (100mM), an activator of class-II aldolases, in the coupled assay. Preincubation of samples in the assay with and without EDTA (5mM) before addition of Fru-1,6-P_2 was used to distinguish the class-I and class-II aldolases. Extracts of the bacterium *Bacillus stearothermophilus* and of the yeast *Saccharomyces cerevisiae* were found to contain only a class II-type aldolase activity, but extracts of the bacterium *Lactobacillus casei* contained roughly equal amounts of class-I and class-II activities. *E. coli* (Crookes' strain) was found to contain about 30% of its total aldolase activity as the class-I enzyme when grown on lactate or pyruvate, but only 5% when grown on

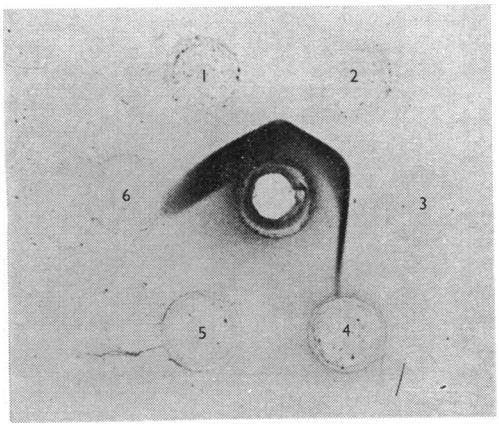


Fig. 8. *Immunodiffusion patterns obtained with aldolase* 1
The central well contained anti-(aldolase 1) serum. Wells 1, 2 and 3 respectively contained undiluted, 3-fold diluted and 9-fold diluted solutions of aldolase 1 (1.7mg/ml). Wells 4, 5 and 6 respectively contained undiluted, 3-fold diluted and 9-fold diluted solutions of aldolase 2 (2.5mg/ml).

glycerol. None of the aldolase activities was affected by the presence of 10mM-citrate in the assay, except for aldolase 1 from *E. coli*.

Anti-(aldolase 1) serum reacted with purified aldolase 1 to give a single precipitin line in immunodiffusion experiments, but did not react with purified aldolase 2 (Fig. 8). Single precipitin lines were also obtained when cell-free extracts of lactate-, pyruvate- or glycerol-grown *E. coli* (Crookes' strain) were used. The weakness of the reaction with glycerol-grown *E. coli* paralleled the low aldolase 1 activity detected in this culture. No reaction was observed with rabbit muscle aldolase, or with cell-free extracts of *L. casei*, *B. stearothermophilus* or *S. cerevisiae*, despite the use of a wide range of concentrations of antiserum and aldolase in each case. Similarly, aldolase 1 showed no reaction with antisera prepared against a number of fish muscle aldolases (S. A. Baldwin and P. A. Benfield, unpublished work).

Discussion

Phenylmethanesulphonyl fluoride was used during the present purification of aldolase 1 in an attempt to avoid proteolysis, a suspected cause of the non-linear Lineweaver–Burk kinetics reported for this enzyme (Stribling & Perham, 1973). This non-linearity now appears to be attributable, at least in part, to contamination of commercial preparations of the substrate (Fru-1,6-P_2) with an activator. However, phenylmethanesulphonyl fluoride evidently has had some beneficial effect, for the molecular weight of the currently isolated enzyme is more than twice that estimated, albeit roughly, for previous preparations. In spite of this finding, both the *N*-terminal residue and the subunit molecular weight are identical with those reported previously for aldolase 1 (Stribling & Perham, 1973; Stribling, 1974). Current estimates of the total molecular weight (approx. 340000) and subunit molecular weight (approx. 35000) suggest a possible decameric structure for aldolase 1, although these estimates, particularly the former, may be subject to considerable error. Decameric proteins are not commonly encountered, but do exist, e.g. arginine decarboxylase from *E. coli* (Boeker & Snell, 1968). In any event, the size of aldolase 1 and the effect of cross-linking reagents on it, indicate that its structure must differ significantly from that of the typical tetrameric class-I enzymes from eukaryotes. In addition, it appears not to resemble other prokaryote class-I enzymes, either in amino acid composition or in size. The class-I aldolase of *Peptococcus aerogenes* is monomeric, with a molecular weight of 33000 (Lebherz & Rutter, 1973), whereas the enzyme from *Lactobacillus casei*, of molecular weight 82000, is a dimer (London, 1974). Only the aldolase from *Mycobacterium smegmatis* resembles the eukaryote aldolases, being tetrameric and of molecular weight 158000 (Bai *et al.*, 1975).

The lack of reaction between anti-(*E. coli* aldolase 1) serum and rabbit muscle aldolase, or class-I aldolase from *Lactobacillus casei*, does not rule out homology among these enzymes, for even the mammalian aldolase isoenzymes, known to be homologous, show no immunological similarity in immunodiffusion experiments (Penhoet *et al.*, 1969). It is probable that only a detailed study of their amino acid sequences will reveal the evolutionary origins of aldolases from such distantly related organisms. Rutter (1964) proposed that the class-I and class-II aldolases arose independently during evolution. The diverse structures of the prokaryote imine-forming aldolases may yet necessitate the introduction of one or more subdivisions for the class-I enzymes. It is also unlikely that aldolases 1 and 2 from *E. coli* share a common ancestor, since their structures and kinetic properties are so different. However, further investigation will be required before the evolutionary origins of these enzymes can be unravelled with certainty.

The discovery of the contamination of commercial samples of Fru-1,6-P_2 led us to observe the activation of aldolase 1 by a variety of compounds. To the best of our knowledge, there are no other reports in the literature of activation of fructose 1,6-bisphosphate aldolases by citrate or other compounds. However, deoxyribose 5-phosphate aldolase from rat liver is activated by citrate and a range of other polycarboxylic acids (Jiang & Groth, 1962). It is essentially inactive in the absence of these compounds (Groth, 1967). As with aldolase 1, the effect of activators

seems to be on the $V_{max.}$ of the enzyme rather than on the K_m for the substrate. The enzyme from *Lactobacillus plantarum* is similarly affected by carboxylic acids (Pricer & Horecker, 1960), but the deoxyribose 5-phosphate aldolase from *E. coli* does not appear to require such compounds for activity (Racker, 1952). The mechanism of the activation remains somewhat obscure. In *L. plantarum* deoxyribose 5-phosphate aldolase, the activators are essential for ^3H exchange between water and the substrate, acetaldehyde, but not for inactivation of the enzyme by borohydride reduction in the presence of substrate (Rosen *et al.*, 1965). Thus it is suggested that the activator is required for formation of the enzyme-bound substrate carbanion. By analogy, a similar role can be envisaged for the activators of aldolase 1, but the mechanism of activation remains to be established. Similarly, it is hard to assess the metabolic significance of the activation phenomenon, particularly since the kinetics have been investigated only in the direction of Fru-1,6-P_2 cleavage, whereas aldolase 1 is likely to have primarily a role in gluconeogenesis (Stribling & Perham, 1973). Alteration of the activity of eukaryote aldolases, brought about by changes in the intracellular ATP and AMP concentrations, has been implicated in the control of glycolysis and gluconeogenesis in rabbit muscle and liver (Morse & Horecker, 1968). Certainly aldolase 1 is very efficient at Fru-1,6-P_2 cleavage when fully activated. However, only a limited range of metabolites has been tested as possible activators, and it would be premature to regard the activation effect as an established mechanism of metabolic control.

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