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Isolation and Characterization of Isocitrate Lyase from a Thermophilic *Bacillus* sp.

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Isocitrate lyase was isolated in homogeneous state from a thermophilic *Bacillus*. The enzyme has a mol.wt. of 180000 and a pI of 4.5 and contains threonine as the *N*-terminal residue. It resembles in size the cognate enzyme from the mesophilic bacterium *Pseudomonas indigofera*, but is smaller than the enzyme from the eukaryotic fungus *Neurospora crassa*. All three lyases are tetramers and similar in amino acid composition, but the thermophile enzyme is distinctive from its mesophilic counterparts in possessing a lower catalytic-centre activity, greater resistance to chemical and thermal denaturation and fewer thiol groups and in being strongly activated by salts. Salt activation, by 0.4M-KCl, is about 3-fold at 30°C and pH6.8 and weakens progressively as the temperature or pH is raised. The activation is probably due to a change in the enzyme conformation caused by the electrolyte modifying the interaction between charged groups or between hydrophobic groups in the protein. The possible significance of the salt activation, of the relative paucity of thiol groups and of the greater resistance to chemical denaturants is discussed. Besides its effect on the $V_{max.}$, KCl produces large increases in the magnitude of several kinetic parameters. A rise in reaction temperature from 30 to 55°C produces a somewhat similar result. In view of these peculiar features, the patterns of inhibition of enzyme activity by compounds such as succinate and phosphoenolpyruvate were examined at 30 and 55°C in the presence and absence of KCl.

Isocitrate lyase (*threo*-D_s-isocitrate glyoxylate-lyase, EC 4.1.3.1) is the first of the two specific enzymes of the glyoxylate cycle, which in effect serves to produce one molecule of a C_4 intermediate of the tricarboxylic acid cycle from two molecules of acetate, and its occurrence has been detected in several micro-organisms and in plants and nematodes (Kornberg, 1966; Reiss & Rothstein, 1974), but not in higher animals such as mammals. It has been purified and well characterized from *Pseudomonas indigofera* (Shiio *et al.*, 1965) and *Neurospora crassa* (Johanson *et al.*, 1974) and less well studied in yeast, *Escherichia coli*, the nematode *Turbatrix aceti* and the alga *Chlorella pyrenoidosa* (Kornberg, 1966; Reiss & Rothstein, 1974; John & Syrett, 1967). A prototrophic thermophilic *Bacillus* sp. capable of using acetate as the sole carbon source for growth and producing the two glyoxylate-cycle enzymes was described by Daron (1967) and a similar isolate was reported by Epstein & Grossowicz (1969). The isocitrate lyase from these two strains is strongly activated by salts, unlike the same enzyme from several mesophilic micro-organisms (Griffiths & Sundaram, 1973; R. M. Chell & T. K. Sundaram, unpublished work), and mutants of the latter thermophile have been isolated that are de-repressed for the two glyoxylate-cycle enzymes (Sundaram, 1973; Chell & Sundaram, 1975). The salt activation, which appeared to be an interestingly peculiar property of the thermophile isocitrate lyase, prompted the isolation in pure state and a detailed characterization of this enzyme. We report here the results of this investigation.

Materials and Methods

Organisms and their growth

The wild-type of the thermophilic *Bacillus* provided by Dr. N. Grossowicz (Hebrew University–Hadassah Medical School, Jerusalem, Israel) was grown aerobically at 55°C in salts medium (Sundaram *et al.*, 1969) supplemented with 50mM-sodium acetate and 0.015% nutrient broth (Oxoid Ltd., Basingstoke, Hants., U.K.) and the mutant PC2NG35, de-repressed for isocitrate lyase and malate synthase (EC 4.1.3.2), was grown similarly in salts medium containing 50mM-sodium acetate and 0.5% nutrient broth. Cells were harvested from late-exponential-phase cultures, washed with 50mM-sodium potassium phosphate buffer, pH7, containing 1mM-EDTA and 0.15M-KCl and stored frozen until used.

Chemicals

Aldolase, chymotrypsinogen, lactate dehydrogenase, malate dehydrogenase, pyruvate kinase and phosphoenolpyruvate (sodium salt) were purchased from Boehringer (London) Ltd., Lewes, East Sussex, U.K., isocitrate dehydrogenase, dithiothreitol, 5,5'-dithiobis-(2-nitrobenzoic acid), phenylmethanesulphonyl fluoride, protamine sulphate, DL-isocitric acid, *threo*-D_s(+)-isocitric acid and thyroglobulin from Sigma (London) Chemical Co. (Kingston upon Thames, Surrey, U.K.) and sodium dodecyl sulphate (specially pure), urea, dansyl-amino acids (chromatographic grade), $(NH_4)_2SO_4$ (specially low in heavy metals) and acrylamide monomer from British Drug Houses (Poole, Dorset, U.K.). Bovine serum albumin was from Calbiochem (Bishops Stortford, Herts., U.K.), guanidine hydrochloride (puriss) was from Fluka A.G. (Fluorochem, Glossop, Derbyshire, U.K.), *NN'*-methylenebisacrylamide and *NNN'N'*-tetraethylenediamine for gel electrophoresis were from Eastman Kodak Co. (Liverpool, U.K.), and Sepharose 6B was from Pharmacia, London W.5, U.K. Other chemicals were obtained from various commercial sources.

Disc gel electrophoresis of native enzymes

Electrophoresis was carried out at pH 8 or 9.2 in 7.5% polyacrylamide gels, prepared in 0.5cm × 7.5cm tubes, at 2mA/gel as described by Gabriel (1971). Gels were stained for protein with 1% (w/v) Amido Black in 7% (v/v) acetic acid and destained by several changes of 7% acetic acid. For the location of isocitrate lyase activity, the gels were incubated for 30–60min at room temperature (20°C) in the dark in a reaction mixture described earlier (Griffiths & Sundaram, 1973); at the end of the incubation the gels were rinsed with 7% acetic acid and stored in this medium in the dark.

Gel electrophoresis of denatured protein

Denatured proteins were electrophoresed in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate, prepared in 0.5cm × 15cm tubes, at 2mA/gel as recommended by Laemmli (1970). The gels were stained for protein with 0.2% Coomassie Brilliant Blue in 50% (v/v) methanol/7% acetic acid and destained in several changes of a 5% methanol/7% acetic acid mixture. The proteins were denatured either by boiling in 1% sodium dodecyl sulphate containing 1% (v/v) 2-mercaptoethanol and 60μg of the proteinase inhibitor phenylmethanesulphonyl fluoride/ml for 2–3min or by boiling in 8M-guanidine hydrochloride containing 1.5% (v/v) mercaptoethanol followed by carboxymethylation (Weber *et al.*, 1972).

Molecular weight of the native enzyme by gel filtration

A column (2.5cm × 38cm) of Sepharose 6B was equilibrated with 50mM-Tris/HCl buffer, pH 7.5, containing 1mM-EDTA and 0.15M-KCl and, after application of the protein sample, was eluted with the same buffer mixture at a flow rate of 10ml/h. The column was calibrated by using appropriate protein markers. The procedure followed was essentially that described by Andrews (1965).

Molecular weight of the native enzyme by sedimentation in the ultracentrifuge

Independent determinations of the molecular weight were carried out by measurement of the sedimentation and diffusion coefficients and by sedimentation-equilibrium centrifugation. Sedimentation coefficients at several protein concentrations in the range 0.5–3.97mg/ml were determined in a Beckman model E ultracentrifuge at 20°C and 56100rev./min with a 12mm double-sector cell and schlieren optics. The enzyme was in 0.1M-sodium/potassium phosphate buffer, pH 7, containing 1mM-EDTA. The $s^0_{20,w}$ value was deduced from these data. The diffusion coefficients in the phosphate/EDTA buffer at 20°C at protein concentrations in the range 1.67–5.99mg/ml were obtained by analysis of the interference patterns by using a double-sector synthetic-boundary centrepiece in an AnJ rotor at 2095rev./min. The $D^0_{20,w}$ value was derived from these data. The molecular weight was obtained by substitution of these s and D values in the Svedberg equation (Schachman, 1959); the partial specific volume was calculated (Cohn & Edsall, 1943) from the amino acid composition. Equilibrium centrifugation was performed at 7447rev./min on a 2mg/ml solution of the protein in 0.1M-phosphate buffer, pH 7, containing 1mM-EDTA and at 9945rev./min and 14290rev./min on a 1.13mg/ml solution of the protein in 0.1M-Tris/HCl buffer, pH 8, containing 1mM-EDTA, in cells with a 12mm double-sector centrepiece. The attainment of equilibrium was ascertained by analysing photographs of the interference patterns taken at hourly intervals for 4–5h (Chervenka, 1969). The molecular weights were calculated from plots of $\log J$ against r^2, where J=absolute fringe number and r=radius in cm (Creeth & Pain, 1967).

Molecular weight of the subunit of isocitrate lyase

The enzyme was denatured by boiling in sodium dodecyl sulphate/mercaptoethanol as described above and the subunits produced were analysed for molecular weight by electrophoresis in polyacrylamide gels (12.5% or 15%) containing 0.1% sodium dodecyl sulphate (Weber *et al.*, 1972) by using appropriate standards.

Amino acid analysis

Samples (2mg) of the pure enzyme were dialysed exhaustively against deionized water and freeze-dried. Each sample was suspended in 2ml of 5.7M-HCl, in duplicate, and hydrolysed by heating in an evacuated sealed tube at 110°C for 24, 48 or 72h. The hydrolysates were evaporated to dryness *in vacuo* and the excess acid was removed by repeated addition of water followed by evaporation to dryness. They were then analysed in a JEOL automatic amino acid analyser. Generally, with the exceptions mentioned below, the values from the 24h, 48h and 72h hydrolysates were averaged to give the amino acid contents. The values for serine and threonine were obtained by linear extrapolation to zero time of hydrolysis. The values for isoleucine and valine, which are slowly released during hydrolysis, were obtained from the results after the 72h hydrolysis. Cysteine and cystine were determined as cysteic acid and methionine was determined as its sulphone in 24h hydrolysates of the performic acid-oxidized enzyme (Hirs, 1956). The cysteine content of the enzyme was independently measured by denaturing the protein with 1% sodium dodecyl sulphate and measuring the free thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence of EDTA (Habeeb, 1972). Tryptophan, which is destroyed during acid hydrolysis, was determined separately by the spectrophotometric method of Edelhoch (1967).

Identification of the N-terminal group in the enzyme

Freeze-dried enzyme (1mg) was denatured by boiling in 0.2M-potassium phosphate, pH8.2, containing 8M-urea for 3min and subjected to dansylation by the procedure of Gros & Labouesse (1969). The dansylated protein was hydrolysed in 5.7M-HCl for 17h at 110°C and the dansylated amino acid in the hydrolysate was identified by two-dimensional t.l.c. on polyamide sheets (Hartley, 1970) by reference to standards of dansylated amino acids.

Purification of isocitrate lyase

Wild-type or mutant PC2 NG35 cells were suspended in 20mM-sodium/potassium phosphate buffer, pH7, containing 1mM-EDTA and 0.15M-KCl, and a cell-free extract was prepared by digestion with lysozyme (100μg/ml) as described previously (Cazzulo *et al.*, 1970). All further operations, unless otherwise indicated, were carried out at about 4°C, and all buffers generally contained 1mM-EDTA. To the cell-free extract was added with stirring 2% (w/v) protamine sulphate (18mg/100mg of protein), and after standing for 15min the precipitate was centrifuged down (30000*g* for 20min) and discarded. The supernatant liquid was fractionated with solid $(NH_4)_2SO_4$, and the protein that was precipitated between 50 and 65% saturation was collected by centrifugation at 30000*g* for 20min and dissolved in 10mM-sodium/potassium phosphate buffer, pH6, and the solution was dialysed for 16h against 100vol. of the same buffer. The dialysed preparation was passed through a column (2cm×12cm) of phosphocellulose (Whatman P11) equilibrated with the phosphate buffer and the column was washed with buffer until no more protein, monitored by the A_{280}, came through. To the effluent, which contained the enzyme activity, was added $(NH_4)_2SO_4$ to 65% saturation. The precipitated protein was collected by centrifugation at 30000*g* for 20min and dissolved in a small volume of 25mM-Tris/HCl buffer, pH7.5, and the solution was dialysed against this buffer overnight. The dialysed protein sample was applied to a column (2.2cm×28cm) of DEAE-cellulose (Whatman DE 52) equilibrated with the Tris/HCl buffer, and after being washed with the same buffer the column was eluted with 800ml of buffer increasing linearly in NaCl concentration from 0 to 0.2M. The active fractions were pooled, $(NH_4)_2SO_4$ was added to 65% saturation and the precipitated protein was centrifuged down at 30000*g* for 20min and dissolved in 10mM-sodium/potassium phosphate buffer, pH6. After dialysis against the same buffer, the protein solution was adjusted to pH5.5 by addition of 0.1M-citric acid solution and fractionated on a column (1.8cm×10cm) of phosphocellulose equilibrated with 10mM-sodium phosphate/citrate buffer, pH5.5. The column was eluted with 400ml of 20mM-sodium/potassium phosphate buffer, pH7, containing a linear gradient of KCl from 0 to 1M. The protein in the most active fractions was precipitated with $(NH_4)_2SO_4$ and was dissolved in, and dialysed for about 18h against, 20mM-sodium/potassium phosphate buffer, pH7.

Assay of protein and enzyme activity

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard, or by the spectrophotometric method of Warburg & Christian (1941). Isocitrate lyase was assayed in the forward direction (isocitrate cleavage) by the continuous spectrophotometric method described by Kornberg (1965) at pH6.8 or by the discontinuous method of McFadden & Howes (1960) at pH6.8 or 8. Imidazole/HCl or sodium/potassium phosphate buffer (25mM) was used in the assays. For assays at temperatures higher than 30°C or in experiments in which the assay temperature was varied, only phosphate buffer was used, because of its relatively small change in pH with temperature. In the continuous assay the temperature of the reaction mixture, which was maintained constant, was measured with a calibrated thermistor. There was good agreement between the values obtained by the two methods at pH6.8 at a given temperature. DL-Isocitrate was routinely the substrate in the assays, but in the

kinetic studies *threo*-D_s-(+)-isocitrate was used. One unit of enzyme catalyses the formation of 1 μmol of glyoxylate/min at 30°C and pH6.8 and specific activity is expressed as enzyme units per mg of protein. For the assay in the reverse direction (formation of isocitrate from glyoxylate and succinate) the reduction of $NADP^+$ concomitant with the oxidation of the isocitrate by isocitrate dehydrogenase was monitored (Williams *et al.*, 1971) at pH6.8 or 8.

Results

Purification of enzyme

A typical purification schedule starting with cells of the wild-type or mutant PC2 NG35 of the thermophilic *Bacillus* is presented in Table 1. The final preparation obtained from either source had the same specific activity. The two preparations were also alike in other properties such as electrophoretic mobility, salt activation, thermostability and K_m for isocitrate, suggesting that the de-repressed mutant produces the same enzyme species as the wild-type but in a markedly greater amount. The purified enzyme was apparently homogeneous as judged by several criteria: it yielded a single protein band on electrophoresis in the native state in polyacrylamide at pH8 or 9.2, and the protein band corresponded to the band revealed when the gel was stained for enzyme activity (Fig. 1); when it was denatured and electrophoresed in sodium dodecyl sulphate/polyacrylamide gel, a single polypeptide species was observed (Fig. 2); its sedimentation profile in the ultracentrifuge displayed a single symmetrical peak. This conclusion is supported by the end-group analysis, which revealed a single amino acid, threonine, at the *N*-terminus. It is noteworthy (Table 1) that isocitrate lyase appears to constitute about 6.5% of the soluble proteins in the wild-type cells; a similarly high proportion has been seen in *P. indigofera* (Shiio *et al.*, 1965) and *C. pyrenoidosa* (John & Syrett, 1967). In mutant PC2 NG35 the content of the lyase is about 16% of the soluble proteins. The purified enzyme can be stored frozen for several months with little loss of activity. Repeated freezing and thawing, however, leads to some precipitation and loss of activity.

Molecular weight of native enzyme

The molecular weight, as determined by gel filtration through a Sepharose 6B column calibrated with the protein markers bovine thyroglobulin (mol.wt. 670000), rabbit muscle pyruvate kinase (mol.wt. 237000), rabbit muscle aldolase (mol.wt.147000), pig muscle lactate dehydrogenase (mol.wt.140000), pig heart malate dehydrogenase (mol.wt. 70000) and malate synthase from the thermophilic *Bacillus* (mol.wt. 62000), was 180000±18000. The sedimentation coefficient, $s^0_{20,w}$, determined from ultracentrifugation runs at several protein concentrations was 9.39S and the diffusion coefficient, $D^0_{20,w}$, was 4.94×10^7 cm^2/s. These values when substituted in the Svedberg equation yielded a mol.wt. of 177296. The values for the molecular weight derived from the three sedimentation equilibrium runs at 7447, 9945 and 14290rev./min were 182000, 183000 and 182000 respectively. At 7447rev./min the plot of log$J$ against r^2 gave some indication of slight polydispersity, possibly due to aggregation (Fig. 3). The average molecular weight of the protein at the bottom of the cells was estimated to be 194000. On the basis of all these values and the molecular weight of the subunit (see below), the native isocitrate lyase was assumed to have a mol.wt. of 180000 for the calculation of its amino acid contents.

Table 1. *Summary of purification of thermophile isocitrate lyase*

For enzyme preparation 60g wet wt. of wild-type cells and 25g wet wt. of mutant PC2 NG35 cells were used. Enzyme activity was assayed at 30°C in imidazole/HCl buffer, pH6.8, by the continuous method.

	Wild-type				Mutant PC2 NG35			
	Protein (mg)	Enzyme activity (units)	Specific activity	Yield (%)	Protein (mg)	Enzyme activity (units)	Specific activity	Yield (%)
1. Cell-free extract	3640	728	0.2	100	1450	711	0.49	100
2. Supernatant fraction after protamine sulphate precipitation	3165	728	0.23	100	1347	718	0.53	101
3. $(NH_4)_2SO_4$ precipitation: dialysed 50–65%-satn. fraction	1152	633	0.55	87	720	589	0.82	83
4. Effluent from phosphocellulose column at pH6	479	618	1.29	85	402	603	1.5	85
5. Fractions from DEAE-cellulose chromatography	122	305	2.5	42	130	302	2.3	43
6. Fractions from phosphocellulose chromatography at pH5.5	69	200	2.9	28	70	207	2.95	29

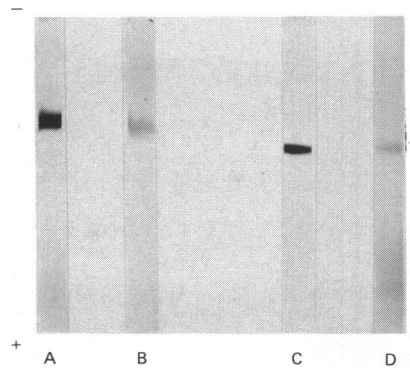

Fig. 1. *Disc electrophoretic analysis of purified thermophile isocitrate lyase in polyacrylamide gel*
The pH of the running buffer for gels A and B was 8.0 and for gels C and D was 9.2. Approx. 30μg of protein was electrophoresed in each gel. Gels A and C were stained for protein and gels B and D were stained for enzyme activity.

Subunit molecular weight

The identification of a single polypeptide species on electrophoresis of the denatured enzyme (Fig. 2) and of threonine and no other amino acid at the *N*-terminus suggests that the thermophile isocitrate lyase contains only one kind of subunit. The molecular weight of this subunit was estimated to be 48000±3300 from its mobility in polyacrylamide (12.5% or 15%) gels containing sodium dodecyl sulphate, related to the mobility of appropriate standards (Fig. 4).

Amino acid composition

The amino acid composition of the thermophile isocitrate lyase is presented in Table 2. For the purpose of comparison, relevant data for the *P. indigofera* and *N. crassa* enzymes are also included. The three enzymes are remarkably similar in their relative amino acid contents, and, with the possible exception of a slight preponderance of glutamic acid/glutamine, there is apparently no unique feature in the composition of the thermophile lyase as compared with the two mesophilic counterparts. The partial specific volume of the thermophile enzyme, calculated from the amino acid composition, was 0.73 ml/g.

Thiol groups

When the thermophile isocitrate lyase was denatured in boiling 1% sodium dodecyl sulphate and treated with 5,5′-dithiobis-(2-nitrobenzoic acid) (Habeeb, 1972), the A_{412} corresponded to nearly three thiol groups per subunit (48000 daltons) of the enzyme (Fig. 5). Without the denaturing treatment one thiol group per subunit reacted with the dithiobis-

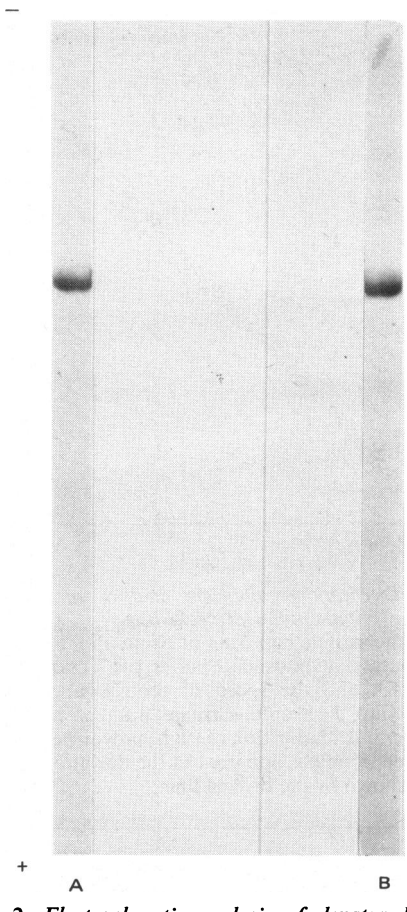

Fig. 2. *Electrophoretic analysis of denatured isocitrate lyase on sodium dodecyl sulphate/polyacrylamide gel*
In each gel 15μg of protein was run. Gel A received the enzyme denatured in boiling 6M-guanidine hydrochloride and then carboxymethylated, and gel B received the enzyme denatured in boiling sodium dodecyl sulphate/mercaptoethanol.

(2-nitrobenzoic acid), suggesting that the remaining thiol groups are less readily accessible, owing to being situated internally in the native structure of the enzyme. At least one of the thiol groups must be essential for enzyme activity, since treatment with 20μM-*p*-chloromercuribenzoate resulted in complete inactivation, which could be reversed by subsequent addition of 1mM-2-mercaptoethanol. Amino acid analysis after oxidation and hydrolysis of the protein revealed three cysteic acid residues per subunit (Table 2), indicating the absence of disulphide groups in the native enzyme. The *P. indigofera* enzyme, which is tetrameric and comparable in size with the thermophile isocitrate lyase, has been reported to have nine available thiol groups and a total of 19–21 half-

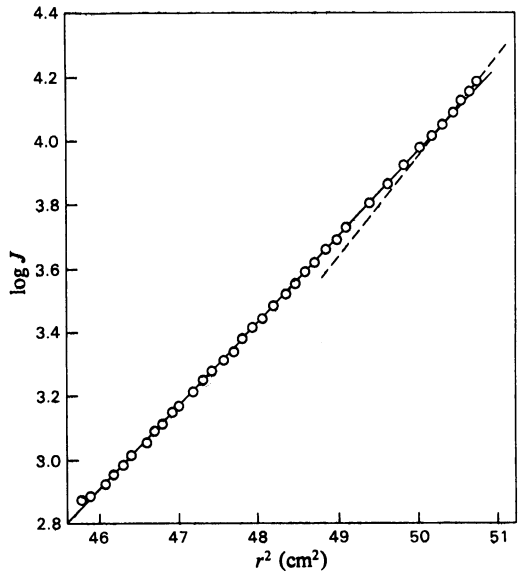

Fig. 3. *Sedimentation-equilibrium analysis of isocitrate lyase in the ultracentrifuge*

The enzyme sample had 2mg of protein/ml in 0.1M-sodium/potassium phosphate buffer, pH7, containing 1mM-EDTA and the speed of centrifugation was 7447rev./min. J=absolute fringe number and r=radius in cm. An indication of slight polydispersity in the enzyme sample is apparent in the deviation from linearity shown by the broken line.

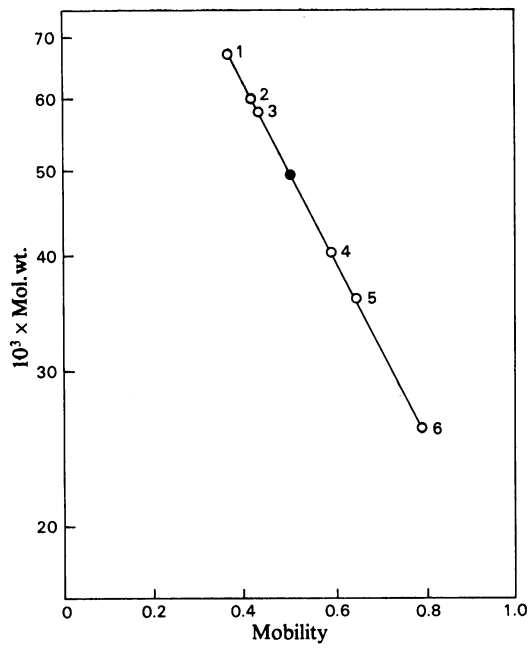

Fig. 4. *Molecular weight of the subunit of thermophile isocitrate lyase*

Denatured proteins were electrophoresed in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulphate. The marker proteins used for the calibration curve were: 1, serum albumin (bovine); 2, malate synthase from the thermophilic *Bacillus*; 3, pyruvate kinase; 4, aldolase; 5, lactate dehydrogenase; 6, chymotrypsinogen A (bovine pancreas). ●, Thermophile isocitrate lyase.

cystine residues per molecule (Shiio *et al.*, 1965; Shiio & McFadden, 1965). The thermophilic *Bacillus* enzyme thus appears to contain significantly (two per subunit) fewer thiol groups than the homologous *Pseudomonas* protein, on the assumption that all the half-cystine residues in the latter enzyme represent cysteine residues.

pH optimum, metal requirement and isoelectric point

The purified enzyme is optimally active at pH8 at 30°C, as shown by assays of its activity in Tris/HCl and imidazole/HCl buffers at a constant ionic strength of 0.075. It has an absolute requirement for Mg^{2+} and the K_m for this metal ion at pH6.8 and 30°C is 0.5mM. Besides fulfilling this essential catalytic function, Mg^{2+} at higher concentrations activates the enzyme as other salts do (see below). The two effects are distinct and the catalytic requirement for Mg^{2+} is not satisfied by the other salts. The isoelectric pH of the thermophile lyase was determined by electrophoresis of the pure enzyme on cellulose acetate strips at 150V for 45min with sodium acetate buffers of various pH values having an I value of 0.1. From a plot of the mobilities of the protein at the different pH values the pI was estimated to be approx. 4.5.

Effect of salt on the catalytic potential and thermostability

An interesting property of the thermophile isocitrate lyase, seen in cell-free extracts and partially purified preparations, is the strong stimulation of its activity by a variety of salts (Griffiths & Sundaram, 1973). These observations are completely reproducible with the homogeneous enzyme. As reported earlier (Griffiths & Sundaram, 1973), the reaction velocity ($V_{max.}$) in the direction of isocitrate cleavage is stimulated about 3-fold by 0.4M-KCl at pH6.8 and 30°C, and this activation becomes progressively weaker as the temperature or pH is raised (Fig. 6). In consequence of the weaker activation at the higher temperatures, the Arrhenius plot for the isocitrate lyase reaction becomes non-linear in the presence of KCl, especially above 45°C, an observation also made earlier with unfractionated cell-free extracts (Griffiths & Sundaram, 1973). Moreover, the slope of the Arrhenius plot decreases appreciably on

Table 2. *Amino acid composition of isocitrate lyase*
The molecular weight of the thermophile enzyme was assumed to be 180000 for the calculation of the number of residues/molecule of the protein. This number was rounded off to the nearest integer. Data for *P. indigofera* and *N. crassa* enzymes were taken from Johanson *et al.* (1974).

	Thermophilic *Bacillus* isocitrate lyase		Mol %	
Amino acid	Mol %	No. of residues/ molecule of enzyme	In *P. indigofera* enzyme	In *N. crassa* enzyme
Aspartic acid	9.66	149	9.66	10.09
Threonine	6.65	102	5.86	5.25
Serine	5.50	85	4.39	4.94
Glutamic acid	15.67	241	11.77	12.30
Proline	2.93	45	3.93	3.83
Glycine	7.37	113	8.61	6.45
Alanine	13.15	202	13.02	12.13
Half-cystine	0.844	13	1.03	1.36
Valine	5.46	84	7.63	5.33
Methionine	1.86	29	2.09	2.36
Isoleucine	5.59	86	4.07	5.15
Leucine	8.00	123	6.85	8.07
Tyrosine	3.61	56	4.10	3.33
Phenylalanine	4.18	64	3.46	2.86
Lysine	6.23	96	5.59	7.91
Histidine	2.28	35	2.28	1.82
Arginine	5.21	80	4.61	4.65
Tryptophan	1.30	20	1.05	2.16

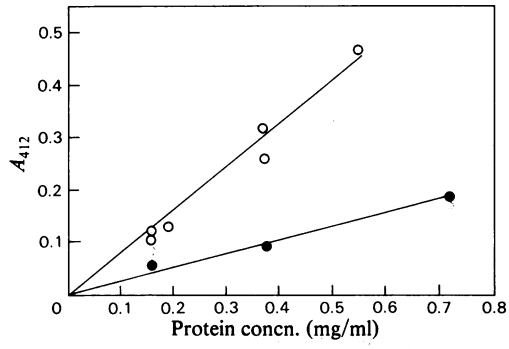

Fig. 5. *Thiol groups in thermophile isocitrate lyase*
The purified enzyme was treated in the native state, or after denaturation with 1% sodium dodecyl sulphate, with 5mM-5,5'-dithiobis-(2-nitrobenzoic acid) and 1mM-EDTA in a total volume of 1ml and the maximum A_{412} attained (after about 20min) was recorded against a suitable reagent blank. ●, Native enzyme; ○, denatured enzyme. From the slopes of the plots and assuming a molar absorption coefficient of 13600 (Habeeb, 1972) for 5-thio-2-nitrobenzoate, the number of thiol groups reacting with 5,5'-dithiobis-(2-nitrobenzoic acid) in 45000 daltons of protein was calculated to be 2.65 for the denatured enzyme and 0.88 for the native enzyme.

inclusion of KCl, and the activation energies calculated from the linear or nearly linear (below 45°C when KCl is present) parts of the plots are 91.5kJ/mol without KCl and 64.2kJ/mol in the presence of 0.4M-KCl. The salt effect on the reaction in the direction of isocitrate synthesis has been examined with the pure enzyme and the pattern of activation at different temperatures and pH values is quite similar to that observed for the cleavage reaction (Fig. 7).

The salt activation, which is exhibited by the homogeneous enzyme, must be an intrinsic property of the thermophile isocitrate lyase related to some special feature of its structure rather than due to an extraneous agent. In the earlier study (Griffith & Sundaram, 1973) isocitrate lyase from a number of mesophilic micro-organisms did not show this strong activation characteristic. Two more mesophilic species, those from *P. indigofera* and *N. crassa*, have now been observed to be not activated by KCl at 30°C. In view of the fact that the salt effect on the thermophile enzyme is weak at temperatures approaching the optimum for the growth of the *Bacillus*, the effect of KCl on the activity of the *P. indigofera* enzyme was also examined at 8°C. There was no activation at this temperature.

KCl also stabilizes the thermophile isocitrate lyase against thermal denaturation. At 55°C in the absence of KCl the enzyme lost 25% of its activity in 2h, but only 10% in the presence of 0.4M-KCl; the half-lives under these two conditions were estimated to be approx. 6 and 15h respectively. The stability of the enzyme in the pure state is considerably greater than in cell-free extracts (Griffiths & Sundaram, 1973), but more similar to that in the intact cell (Coultate *et al.*, 1975). At higher temperatures KCl has little effect on the thermostability of the pure enzyme. Thus at 57°C 40% of the activity was lost in 1h irrespective of whether KCl was present or not, and at 60°C the half-lives were 3min without KCl and 2.2min with 0.4M-KCl.

Kinetic parameters and their dependence on salt and temperature

The K_m for *threo*-D$_s$(+)-isocitrate in the cleavage reaction in 25mM-imidazole/HCl buffer, pH6.8, at 30°C is 0.02mM. When 0.4M-KCl is present, the K_m increases 10-fold concomitantly with an approximately 3-fold increase in $V_{max.}$. In a phosphate buffer system without KCl the K_m is nearly 3 times that in the imidazole system, but the $V_{max.}$ is unchanged, and in the presence of 0.4M-KCl the K_m as well as $V_{max.}$ is the same in phosphate as in imidazole/HCl

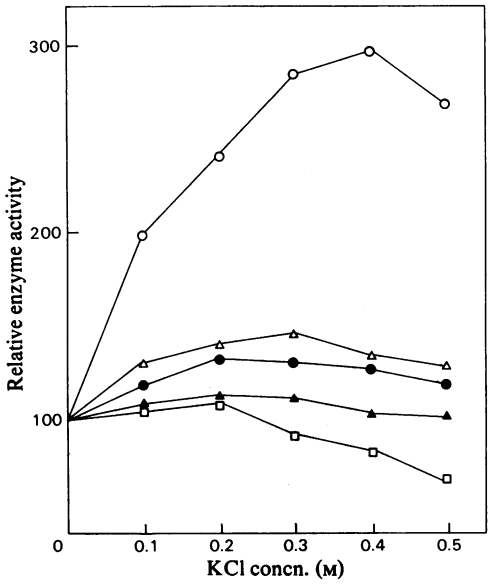

Fig. 6. *Effect of KCl on the forward reaction (isocitrate cleavage) catalysed by thermophile isocitrate lyase*
Enzyme assays were performed in 25mM-sodium/potassium phosphate buffer, pH6.8 (open symbols), by the continuous spectrophotometric method or in the same buffer, pH8 (closed symbols), by the discontinuous method, with the addition of KCl as indicated. The temperatures of the assays were: ○, ●, 30°C; △, ▲, 45°C; □, 54.5°C. An appropriate amount of enzyme in the range 0.5–5 milliunits was assayed in a 1ml system. The activity in the system without KCl is represented as 100.

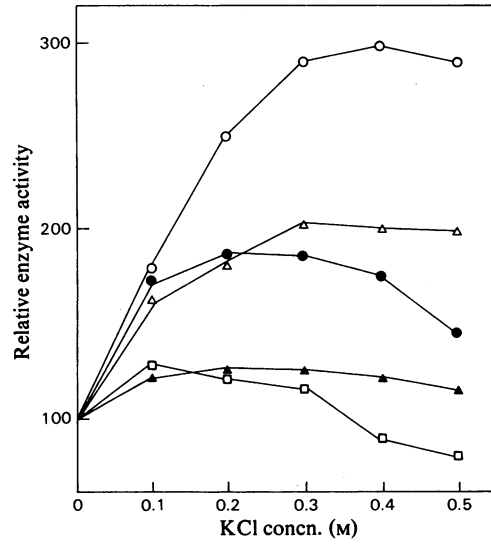

Fig. 7. *Effect of KCl on the backward reaction (isocitrate formation) catalysed by thermophile isocitrate lyase*
Enzyme assays were performed in 25mM-sodium/potassium phosphate buffer, pH6.8 (open symbols) or pH8 (closed symbols), with the addition of KCl as indicated. The temperatures of the assays were: ○, ●, 30°C; △, ▲, 45°C; □, 54.5°C. An appropriate amount of enzyme in the range 2–20milliunits was assayed in a 1ml system. The activity in the system without KCl is represented as 100.

buffer (Table 3). A higher K_m for isocitrate in phosphate buffer has been observed with NADP-dependent isocitrate dehydrogenase from *Bacillus stearothermophilus* (Howard & Becker, 1970). For the reverse (condensation) reaction the K_m values for succinate and for glyoxylate, determined in imidazole/HCl, pH6.8, increase 10-fold and 3-fold respectively on addition of 0.4M-KCl (Table 3). An appreciably higher K_m for isocitrate in the cleavage reaction, performed in phosphate buffer, is also seen when the reaction temperature is raised and a further increase results at the high temperature when KCl is present during the assay (Table 3).

Several compounds inhibitory to *P. indigofera* isocitrate lyase are effective inhibitors also of the thermophile enzyme. However, in the latter system the magnitude of the inhibition constants, like that of the K_m values for the substrates, is greatly dependent on the temperature of the assay and on whether or not KCl is present (Table 4). For example, the inclusion of 0.4M-KCl raises the constant for phos-

Table 3. *Michaelis constants (K_m) for substrates in the thermophile isocitrate lyase reaction*
Assays of the cleavage reaction were in 25mM-imidazole/HCl or 25mM-phosphate buffer, pH 6.8, by the continuous method. Assays of the condensation reaction were in 25mM-imidazole/HCl buffer, pH6.8. KCl was either absent (–) or present (+).

Substrate	Buffer system	Temperature (°C)	KCl (0.4M)	K_m (mM)
Cleavage reaction				
Isocitrate	Imidazole	30	–	0.02
Isocitrate	Imidazole	30	+	0.20
Isocitrate	Phosphate	30	–	0.059
Isocitrate	Phosphate	30	+	0.20
Isocitrate	Phosphate	55	–	0.10
Isocitrate	Phosphate	55	+	0.77
Condensation reaction				
Succinate	Imidazole	30	–	0.054
Succinate	Imidazole	30	+	0.54
Glyoxylate	Imidazole	30	–	0.047
Glyoxylate	Imidazole	30	+	0.142

Table 4. *Inhibition constants (K_i) in the thermophile isocitrate lyase reaction and the patterns of inhibition*
Assays were performed as indicated in Table 3 with the inhibitors present as required and the steady-state reaction rates were used to construct the plots. Inhibition constants were calculated either from the linear replots (see insets in Figs. 8 and 9) or from the equations for competitively, non-competitively and uncompetitively inhibited enzyme reactions.

Inhibitor tested	Buffer system	Temperature (°C)	KCl (0.4M) present or not	K_i (mM)	Inhibition pattern
Cleavage reaction					
Phosphoenolpyruvate	Imidazole	30	−	0.008	Linearly uncompetitive with isocitrate
Phosphoenolpyruvate	Imidazole	30	+	0.095	Linearly uncompetitive with isocitrate
Phosphoenolpyruvate	Phosphate	30	−	0.028	Linearly uncompetitive with isocitrate
Phosphoenolpyruvate	Phosphate	30	+	0.10	Linearly uncompetitive with isocitrate
Phosphoenolpyruvate	Phosphate	55	−	0.08	Linearly uncompetitive with isocitrate
Phosphoenolpyruvate	Phosphate	55	+	0.135	Linearly uncompetitive with isocitrate
Succinate	Imidazole	30	−	—	Mixed: non-linear replots
Succinate	Imidazole	30	+	1.85	Linearly non-competitive with isocitrate
Succinate	Phosphate	30	−	—	Mixed: non-linear replots (see Fig. 9)
Succinate	Phosphate	30	+	1.85	Linearly non-competitive with isocitrate
Succinate	Phosphate	55	−	0.90	Linearly non-competitive with isocitrate
Succinate	Phosphate	55	+	2.3	Linearly non-competitive with isocitrate
Condensation reaction					
Phosphoenolpyruvate	Imidazole	30	−	0.007	Linearly competitive with succinate Linearly uncompetitive with glyoxylate
Phosphoenolpyruvate	Imidazole	30	+	0.155	Linearly competitive with succinate
Itaconate	Imidazole	30	−	0.0004	Linearly competitive with succinate
Itaconate	Imidazole	30	+	0.0055	Linearly competitive with succinate
Maleate	Imidazole	30	−	0.006	Linearly competitive with succinate
Malonate	Imidazole	30	−	0.25	Linearly competitive with succinate

phoenolpyruvate in the cleavage reaction 12-fold in imidazole/HCl buffer, pH6.8, at 30°C. The inhibition constant in phosphate buffer, pH6.8, at 30°C is significantly higher than in imidazole buffer, but increases some 4-fold on addition of 0.4M-KCl; at 55°C in phosphate buffer, pH6.8, 0.4M-KCl causes an approx. 2-fold increase in the value of the inhibition constant. In the absence of KCl a rise of temperature from 30 to 55°C results in a nearly 3-fold increase in this constant in phosphate buffer, pH6.8. The inhibition constants for phosphoenolpyruvate in the condensation reaction and the constants for several other compounds and the patterns of inhibition by the various inhibitors under a variety of conditions are also shown in Table 4. Representative plots illustrating the inhibition patterns are presented in Figs. 8 and 9. It is worth noting that concomitant with an increase in the K_m for isocitrate, due to the presence of KCl or to a rise in temperature, there is an alleviation of the substrate inhibition of enzyme activity, since a higher isocitrate concentration is required to saturate the enzyme.

Denaturability of thermophile isocitrate lyase

The results summarized in Table 5 show that significant denaturation of the enzyme occurs at 25°C only with relatively high concentrations of guanidine hydrochloride or with even higher concentrations of urea. It must be pointed out that in the assay system the denaturant was diluted 20-fold and therefore some re-activation possibly resulted between the start of the assay and the establishment of a steady reaction rate; this time interval was as long as 7–8min when guanidine hydrochloride was used at the higher concentrations. After the treatment with 8M-urea or 2.4M-guanidine hydrochloride, dialysis against 20mM-phosphate buffer, pH7, containing 1mM-EDTA effected complete recovery of the enzyme activity. After the treatment with 3.6M-guanidine hydrochloride dialysis restored 86% of the original enzyme activity. The higher (over 100%) activity seen with the lower concentrations of guanidine hydrochloride is presumably due to activation analogous to that produced by KCl and other salts superimposed on a low degree of denaturation. Although this activation as well as the time lag between the start of the enzyme assay and the establishment of the steady reaction rate probably underestimated the extent of denaturation, it is clear that the thermophile isocitrate lyase is markedly more resistant to the denaturants than is the *P. indigofera* enzyme (McFadden *et al.*, 1968). Incubation of the *Pseudomonas* enzyme for 10min at 25°C with 0.8M-guanidine hydrochloride produced 88% inactivation, and 74% of the original activity could be regained by

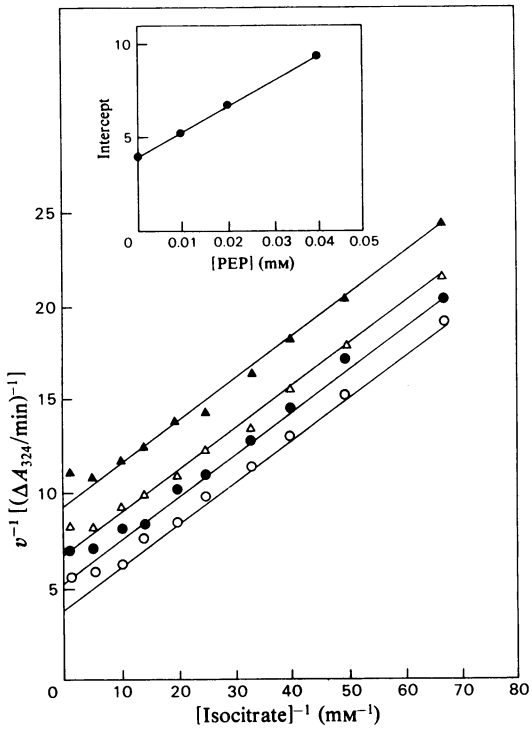

Fig. 8. *Inhibition of the isocitrate lyase cleavage reaction by phosphoenolpyruvate in 25mM-phosphate buffer pH6.8 at 30°C*

Enzyme activity was assayed spectrophotometrically with 0 (○), 0.01mM- (●), 0.02mM- (△) and 0.04mM- (▲) phosphoenolpyruvate PEP. The inset shows a replot of the intercepts against inhibitor concentrations.

dialysis. Complete inactivation resulted with 1.6M-guanidine hydrochloride and an irreversible inactivation of 96% occurred with 3M-urea.

Discussion

Our study establishes that the thermophilic *Bacillus* isocitrate lyase is a tetramer, like the mesophilic *P. indigofera* and *N. crassa* enzymes, which are the only ones to have been similarly characterized. The identification of a single amino acid, threonine, at the *N*-terminus of the thermophile enzyme further suggests that the subunits are probably identical. The enzyme from *Neurospora*, a eukaryote, with a mol.wt. of 270000 is, however, an appreciably larger protein. A comparison between the thermophile enzyme and the homologous mesophilic enzymes with respect to a number of properties is presented in Table 6. Although the thermophile enzyme bears

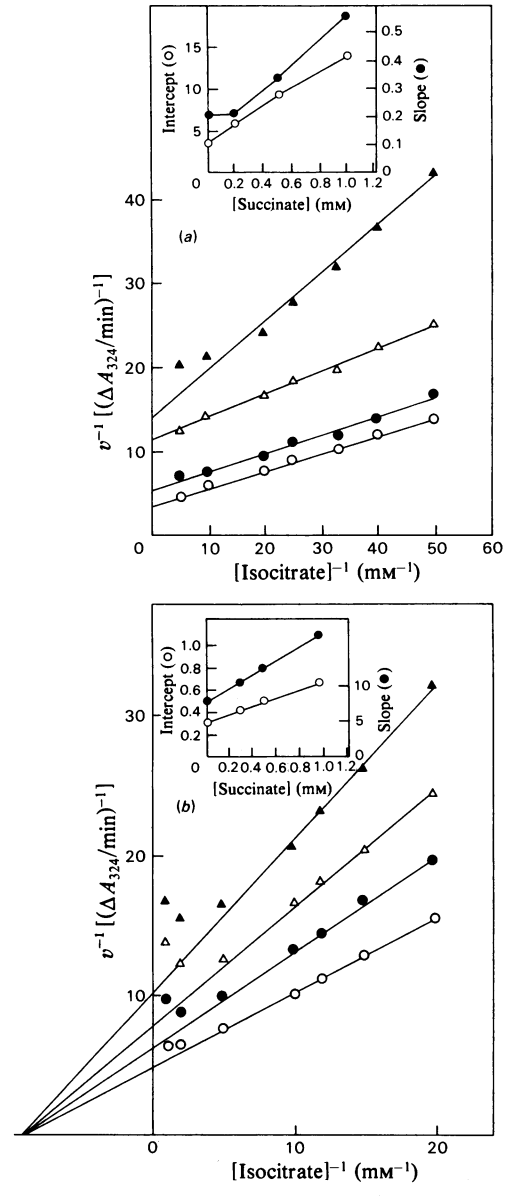

Fig. 9. *Inhibition of the isocitrate lyase cleavage reaction by succinate in 25mM-phosphate buffer, pH6.8*

Enzyme activity was assayed spectrophotometrically at 30°C (*a*) and at 55°C (*b*). Succinate concentrations (mM) were 0 (○), 0.1 in (*a*) and 0.3 in (*b*) (●), 0.5 (△) and 1.0 (▲). Insets show replots of the slopes and intercepts against inhibitor concentrations.

remarkable resemblance to the mesophile ones in several respects, it is distinctive in possessing such characteristics as lower catalytic efficiency (catalytic-centre activity) at 30°C, greater resistance to thermal

and chemical denaturation, fewer thiol groups and strong activation by salt at the lower temperatures (30°C and below).

Sufficient comparative data have not apparently been examined to determine whether thermophile enzymes are necessarily endowed with less catalytic activity than are the cognate mesophile enzymes. We have observed in this laboratory quite low catalytic-centre activity values at 30°C with certain thermophile malate dehydrogenases, especially those from extremely thermophilic bacteria, and a thermophile aldolase has been reported to be quite inactive at temperatures below 60°C (Freeze & Brock, 1970). It is reasonable to speculate that the high resistance to thermal and chemical denaturation of the thermophile isocitrate lyase may stem from the same structural peculiarity. However, Barnes *et al.* (1971) observed that aldolase from two thermophilic *Clostridium* species was as susceptible to denaturation by urea and guanidine as the same enzyme from a mesophilic *Clostridium*. Thus, as with catalytic-centre activity, it is not unequivocally established whether thermophile enzymes are distinctive from their mesophilic counterparts in susceptibility to chemical denaturation. The relative paucity of thiol groups in the thermophile isocitrate lyase (Fig. 5, Table 6) in comparison with the *Pseudomonas* and *Neurospora* (Johanson *et al.*, 1974) enzymes, which may not be readily apparent from gross amino acid compositions (Table 2), could be a relevant feature of its thermostable structure. Although there are other cases of a similar distinction between homologous thermophile and mesophile proteins (Singleton & Amelunxen, 1973), it may not be a universal criterion of protein thermostability in thermophiles (Devanathan *et al.*, 1969). All these observations highlight the currently favoured view that the same general mechanism of thermostabilization will not be applicable to all thermophile systems.

As pointed out earlier (Griffiths & Sundaram, 1973), strong salt activation apparently is a charac-

Table 5. *Denaturation of thermophile isocitrate lyase by urea and guanidine hydrochloride*

Purified isocitrate lyase (100 μg) was incubated for 15 min at 25°C in 1 ml of a mixture (pH 7) containing 20 mM-phosphate, 1 mM-EDTA, 0.1 mM-dithiothreitol and the denaturant at the concentration indicated. A portion (50 μl) was taken for assay at pH 6.8 and 30°C in the spectrophotometric system (1 ml). From the steady reaction rate in this system, the activity of the denaturant-treated enzyme was calculated and expressed as % of the activity of the untreated enzyme.

Urea		Guanidine hydrochloride	
Concn. of denaturant (M)	Activity remaining (%)	Concn. of denaturant (M)	Activity remaining (%)
3	100	0.6	121
4	89	1.2	142
5	82	1.8	120
6	80	2.4	63
8	69	3.0	39
—	—	3.6	25
—	—	4.2	15

Table 6. *Comparative survey of thermophile and mesophile isocitrate lyases*

Data for the *P. indigofera* and *N. crassa* enzymes, except those on the salt effect, were collected from McFadden *et al.* (1968) and Johanson *et al.* (1974); parameters $H\phi_{av.}$, NPS and ρ for these enzymes were calculated by us from their published amino acid compositions. The salt effect on the two enzymes was tested in dialysed extracts of the organisms grown in salts media containing acetate as carbon source.

Parameter	Isocitrate lyase from Thermophilic *Bacillus*	*Pseudomonas indigofera*	*Neurospora crassa*
Mol.wt.	180000	206000	270000
Subunit mol.wt.	48000	48000	67000
No. of subunits	4	4	4
Catalytic-centre activity at 30°C (molecules of isocitrate cleaved/s per catalytic centre)	24 at pH 8	122 at pH 7.7	72 at pH 6.8
No. of thiol (half-cystine) groups/molecule	12	19–21	33
Average hydrophobicity ($H\phi_{av.}$)	1.02	0.96	1.09
Frequency of non-polar side-chains (NPS)	0.294	0.289	0.308
Ratio of polar volume to non-polar volume (ρ)	1.18	1.06	1.09
Salt (0.4 M-KCl) effect at 30°C and pH 6.8	3-fold activation	18% inhibition	98% inhibition
Urea effect (25°C)	Partial inactivation by 8 M-urea in 15 min; complete re-activation upon removal of urea	96% irreversible inactivation by 3 M-urea in 10 min	—

teristic property of the *Bacillus* thermophile isocitrate lyase which is not shared by many mesophile isocitrate lyases that we have examined and which the thermophile enzyme probably acquired concomitantly with thermostability during evolution. Salt presumably causes a change in the conformation of the enzyme, possibly by interacting with charged groups and/or by accentuating interactions between hydrophobic groups in a crucial part of the protein molecule. The marked alterations in a number of kinetic parameters of this enzyme produced by salt are consistent with this interpretation. It was expected that such conformational change might perhaps be manifested in the absorption spectrum of the protein. However, no alteration in the absorption characteristics could be demonstrated in the presence of 0.4M-KCl in the range 258–300nm. The parameters $H\phi_{av.}$ and NPS (Table 6) are thought to be indices of the non-polarity or hydrophobicity, and ρ (Table 6) is considered to be an index of the polarity of the protein molecule (Bigelow, 1967; Fisher, 1964; Waugh, 1954). These parameters, calculated from amino acid composition, are given in Table 6 for the thermophile isocitrate lyase and the cognate enzymes from *P. indigofera* and *N. crassa*. As in several other systems (Singleton & Amelunxen, 1973), no gross overall preponderance of polar or non-polar residues is evident in the thermophile isocitrate lyase molecule. Nevertheless, this does not preclude the possibility of these groups being situated strategically in the protein molecule. The weakening of the salt activation at higher temperatures may be interpreted as indicating a change in the conformation of the enzyme with temperature, which possibly separates the groups whose interaction is presumed to be strengthened by salt at the lower temperatures. Numerous instances have been reported of thermophile enzymes changing their conformation at high temperature around 55°C (Singleton & Amelunxen, 1973). The possibility of a change in the state of aggregation of thermophile isocitrate lyase in the presence of salt may be ruled out by our finding that KCl causes little change in the sedimentation velocity of the protein in the ultracentrifuge. It is now generally recognized, contrary to earlier expectations, that the greater thermostability of thermophile proteins is probably based on subtle rather than gross structural peculiarities and that a single general mechanism may not account for the stability of all such proteins. Stabilization by hydrophobic clusters, by salt linkages, by hydrogen bonds and by strong specific binding of metal ions leading to a more resistant conformation have been variously invoked to explain the thermostability (Singleton & Amelunxen, 1973; Perutz & Raidt, 1975). Our investigation of the other glyoxylate-cycle enzyme, malate synthase, from the thermophilic *Bacillus* shows that it is a monomeric protein which is markedly labilized by an increase in ionic strength brought about by the addition of KCl and therefore, as suggested by Perutz & Raidt (1975), owes its thermostability in large part to salt bridges in its structure. The thermophile isocitrate lyase, however, is not labilized by the addition of KCl. If its salt activation is an indication of the presence of specific, strategically located, hydrophobic groups, it is likely that these groups rather than salt bridges contribute to the thermostabilization of the lyase.

The thermophile isocitrate lyase resembles its *Pseudomonas* homologue in several kinetic characteristics: phosphoenolpyruvate inhibition, which is competitive with succinate and uncompetitive with isocitrate and glyoxylate, and inhibition by itaconate, maleate and malonate competitive with succinate. Moreover, there is a good deal of similarity between the two systems in the pattern of succinate inhibition. The *Pseudomonas* enzyme is inhibited by succinate non-competitively with respect to isocitrate at 30°C. In the thermophile system, in view of its peculiar nature, we have examined the succinate inhibition at 30 and 55°C and in the presence and absence of KCl. At 30°C with KCl present and at 55°C with or without the salt the inhibition is linearly non-competitive with isocitrate. However, at 30°C without KCl the inhibition appears to be mixed and characterized by non-linear replots of both the slope and intercept against inhibitor concentration (Table 4 and Fig. 9). This may indicate that the kinetic mechanism at the lower temperature in the absence of salt is not the same as that at high ionic strength or at high temperature, possibly because the enzyme exists in a different conformation. Non-competitive inhibition by succinate and the observed patterns of inhibition by itaconate and phosphoenolpyruvate are consistent with an ordered Uni Bi mechanism of isocitrate cleavage in which succinate obligatorily leaves the reaction complex before glyoxylate, and itaconate and phosphoenolpyruvate bind at the succinate-specific site (Williams *et al.*, 1971). It may be significant that at high ionic strength or at high temperature, both physiological conditions that must prevail inside the growing thermophilic bacterial cell, succinate inhibition becomes linearly non-competitive. The large increases in the values of a number of kinetic parameters caused by the inclusion of salt or by a rise in temperature are another interesting feature of the thermophile enzyme. It is noteworthy that the increase in the catalytic potential ($V_{max.}$) produced by salt at the lower temperatures is accompanied by a weaker interaction of the enzyme with its substrates, as indicated by the larger K_m values.

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