Structural Basis of the Thermostability of Monomeric Malate Synthase from a Thermophilic *Bacillus*

R. M. CHELL AND T. K. SUNDARAM*

Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, England

Malate synthases from a thermophilic *Bacillus* and *Escherichia coli* have been isolated in a high state of purity. Molecular weights of these two proteins determined in the native state and after denaturation in sodium dodecyl sulfate-mercaptoethanol show that the enzymes are monomeric. This conclusion is supported, for the thermophile enzyme, by the result of an electrophoretic analysis of that protein after treatment with dimethylsuberimidate and denaturation. The thermophilic *Bacillus* malate synthase is considerably more thermostable than its mesophilic counterparts from *E. coli*, *Bacillus licheniformis*, and *Pseudomonas indigofera*. It is, however, markedly labilized by an increase in the ionic strength of the medium brought about by the addition of 0.2 M potassium chloride or in pH above 9. Increased ionic strength has little effect on the thermostability of the mesophilic bacterial malate synthases. These observations provide strong support for the idea that monomeric proteins in thermophiles owe their unusual heat stability to the presence of salt bridges in their tertiary structure.

It is well established that proteins from thermophilic microorganisms are endowed with considerably greater innate thermostability than their counterparts from mesophilic organisms. The molecular basis of this stability is unclear but is now presumed to reside in some subtle structural peculiarity, which may vary from one protein or class of proteins to another (10, 20). From a stereochemical examination of the structures of ferredoxins isolated from mesophilic and thermophilic bacteria, Perutz and Raidt (18) have suggested that in ferredoxin and other monomeric proteins from thermophiles the extra energy of stabilization can be provided without disturbance of the tertiary structure through a small number of extra salt bridges on the molecular surface. No experimental confirmation of this hypothesis has been reported, to our knowledge, with ferredoxin or any other monomeric protein. Our investigation of malate synthase (L-malate glyoxylate-lyase [coenzyme A-acetylating], EC 4.1.3.2), a glyoxylate cycle enzyme (12), from a thermophilic *Bacillus* reveals that it is a monomeric protein of exceptional thermostability and that this stability can be attributed in large part to the presence of salt bridges in its structure. We present here the results of this study.

MATERIALS AND METHODS

Organisms and growth conditions. A prototrophic thermophilic *Bacillus* (wild type) (24) and a mutant (PC2 NG35) (23) derepressed for malate synthase were grown at 55°C in a salts medium (24) supplemented with 50 mM sodium acetate and nutrient broth (0.01% for the wild type and 0.5% for the mutant). A mutant of *Escherichia coli* derepressed for malate synthase, provided by H. L. Kornberg, and *Bacillus licheniformis* A5 were grown at 37°C in salts media (22, 24) supplemented with acetate, and *Pseudomonas indigofera*, obtained from B. A. McFadden, was grown at 30°C in acetate-supplemented salts medium (15). All cultures were grown under aerobic conditions.

Chemicals. Acetyl coenzyme A was prepared from coenzyme A and acetic anhydride, and its concentration was determined as described previously (4). Isocitrate lyase was purified from the thermophilic *Bacillus* by a procedure outlined earlier (5). The sources of the other chemicals were as follows: acetic anhydride, tris(hydroxymethyl)aminomethane (Tris) base, and glycine (Analar grade), ethylenediaminetetraacetate (EDTA), acrylamide monomer, sodium dodecyl sulfate (SDS) (specially pure), ammonium sulfate (specially low in heavy metals), Nitro Blue Tetrazolium, and amido black 12B from British Drug Houses Ltd.; *N,N'*-methylene bisacrylamide and *N,N,N',N'*-tetramethylethylenediamine for polyacrylamide gels from Eastman Kodak Co.; bovine serum albumin and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) from Calbiochem; Sephadex G-200 from Pharmacia; dimethylsuberimidate dihydrochloride from Aldrich Chemical Co.; coenzyme A, nicotinamide adenine dinucleotide, glutamate dehydrogenase, catalase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, malate dehydrogenase, ovalbumin, cytochrome *c*, pyruvate kinase, chymotrypsinogen A, and aldolase from

Boehringer; lysozyme, phenylmethylsulfonyl fluoride, glyoxylic acid, 2-mercaptoethanol, phenazine methosulfate, protamine sulfate, Coomassie brilliant blue R, and adenylate kinase from Sigma. Hydroxyapatite was prepared by the method of Atkinson et al. (2). All other chemicals were purchased from various commercial sources.

Preparation of cell-free extracts. Cell-free extracts of the thermophilic *Bacillus* and of *B. licheniformis* were prepared as described previously (23, 24). To prepare a cell-free extract of *E. coli*, cells were suspended in 3 volumes of 5 mM Tris-chloride buffer (pH 8) containing 10 mM EDTA and incubated with lysozyme (250 μg/ml) for 15 min at 30°C. The lysozyme-treated suspension was passed through a French press (3,000 lb/in^2 pressure), diluted with an equal volume of Tris-chloride buffer containing 1 mM MgCl$_2$, and then treated with an Ultra-Turrex tissue homogenizer for about 10 s. The homogenate was centrifuged in an MSE angle rotor at 30,000 × *g* and 4°C for 30 min to yield the cell-free extract. *P. indigofera* cells, suspended in ice-cold 5 mM Tris-chloride buffer (pH 8) containing 1 mM MgCl$_2$, were disrupted by an ultrasonic drill (Measuring and Scientific Equipment Ltd.), and the homogenate was centrifuged at 30,000 × *g* for 30 min at 4°C.

Gel electrophoretic analysis of denatured malate synthase. Malate synthases isolated from the thermophilic *Bacillus* and *E. coli* were denatured by treatment for 4 min in boiling 1% SDS containing 1% 2-mercaptoethanol and 0.3 mg of the protease inhibitor, phenylmethylsulfonyl fluoride, per ml and subjected to electrophoresis in 10% polyacrylamide gels supplemented with 0.1% SDS (13), which were prepared in cylindrical tubes (0.5 by 15 cm). Electrophoresis was performed at 3 mA per gel for 2.5 h, and the gels were stained for protein with 0.2% Coomassie brilliant blue in 50% (vol/vol) methanol–7% (vol/vol) acetic acid and destained of excess dye in 5% methanol–7% acetic acid. The molecular weights of the polypeptide species visualized in the gels were determined from their mobilities relative to the mobilities of appropriate standards (26); the standards used were bovine serum albumin, pyruvate kinase and aldolase (rabbit muscle), thermophilic *Bacillus* isocitrate lyase (5), malate dehydrogenase (pig heart), and bovine chymotrypsinogen A.

To confirm its suspected monomeric structure, the thermophilic *Bacillus* malate synthase (1 mg/ml) was treated with the cross-linking reagent, dimethylsuberimidate (2 mg/ml) (6), in 0.2 M triethanolamine-hydrochloride (pH 8.5) for 72 h at room temperature. The treated enzyme was then denatured in SDS-mercaptoethanol and subjected to electrophoresis in SDS-polyacrylamide.

Polyacrylamide gel electrophoresis of native enzyme. The procedure employed was based on the method of Ornstein and Davis (17). The acrylamide concentration for the gels (0.5 by 7.5 cm) was 7.5% (wt/vol), and the pH of the running gel was 9.2. Electrophoresis was performed at 2 mA per gel for 2 h, and the gels were stained for either protein or malate synthase activity. Staining for protein was done with 1% (wt/vol) amido black in 7.5% (vol/vol) acetic acid, and excess dye was removed with 7.5% acetic

acid. Gels were stained for enzyme activity in the following mixture: Tris-chloride (pH 8), 0.1 M; MgCl$_2$, 10 mM; acetyl coenzyme A, 0.2 mM; sodium glyoxylate, 2 mM; nicotinamide adenine dinucleotide, 0.3 mg/ml; phenazine methosulfate, 0.2 mg/ml; Nitro Blue Tetrazolium, 0.4 mg/ml; malate dehydrogenase (pig heart), 0.2 IU/ml. After incubation at 37°C in the dark for a suitable period, the gels were washed and stored in 7% acetic acid.

Determination of native molecular weights of malate synthases. The gel filtration method of Andrews (1) was used. A column (2.5 by 38 cm) of Sephadex G-200 was equilibrated with 50 mM Tris-chloride buffer (pH 8) containing 1 mM EDTA, 2 mM MgCl$_2$, and 0.1 M KCl. After application of the protein sample, the column was eluted with the same buffer mixture at a flow rate of approximately 18 ml/h. Suitable protein markers were used to calibrate the column, and they were determined in the effluent fractions by the following methods: glutamate dehydrogenase (beef liver) (EC 1.4.1.3) by enzymic assay (21); catalase (beef liver) (EC 1.11.1.6) by absorbance at 407 nm; isocitrate lyase (thermophilic *Bacillus*) (EC 4.1.3.1) by enzymic assay (10); glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle) (EC 1.2.1.12) by absorbance at 220 nm (1); lactate dehydrogenase (hog muscle) (EC 1.1.1.27) and malate dehydrogenase (pig heart) (EC 1.1.1.37) by enzymic assay (11, 16); bovine serum albumin, ovalbumin, and adenylate kinase (pig muscle) by absorbance at 230 nm (1); and cytochrome *c* (horse heart) by absorbance at 412 nm.

Malate synthase assay. Two alternative systems were used. One system contained in 1 ml: Tris-chloride (pH 8), 50 μmol; MgCl$_2$, 10 μmol; acetyl coenzyme A, 0.05 μmol; DTNB, 0.2 μmol; enzyme and glyoxylate, 1 μmol. The initial rate of increase in absorbance at 412 nm following the addition of glyoxylate at 30°C was measured with a recording spectrophotometer. The second system differed from the first system in not containing DTNB. The initial rate of decrease in absorbance at 232 nm following the addition of glyoxylate was measured (8). One unit of enzyme catalyzes the formation of 1 μmol of coenzyme A per min, and specific activity is expressed as units per milligram of protein.

Protein. Protein was determined in cell-free extracts by the method of Lowry et al. (14) using bovine serum albumin as the standard and in purified preparations by the method of Warburg and Christian (25).

Purification of thermophilic *Bacillus* malate synthase. All operations, unless indicated otherwise, were carried out at 4°C, and the buffers generally contained 1 mM EDTA. The cell-free extract, prepared from the wild type or mutant PC2 NG35 of the thermophilic *Bacillus,* was treated with protamine sulfate (18 mg per 100 mg of protein), and after standing for 15 min the precipitate was spun down and discarded. The supernatant liquid was fractionated with solid ammonium sulfate; the protein precipitated between 50 and 65% saturation levels was dissolved in 10 mM sodium potassium phosphate buffer (pH 6) and dialyzed against 100 volumes of this buffer. The dialyzed preparation was passed through a column (2 by 12 cm) of phosphocellulose (Whatman P11) equilibrated with the phosphate buffer, and the column

was washed with buffer. All the effluent fractions containing protein were pooled, and the protein in them was precipitated with ammonium sulfate (430 g/liter). The precipitate was collected by centrifugation and dissolved in 25 mM Tris-chloride (pH 7.5), and the solution was dialyzed against 100 volumes of the same buffer. The dialyzed solution was fractionated on a diethylaminoethyl (DEAE)-cellulose (Whatman DE 52) column (2.2 by 28 cm) equilibrated with the Tris-chloride buffer. The column was eluted with 800 ml of buffer containing a linear gradient of NaCl from 0 to 0.2 M to release the malate synthase. The protein in the active fractions was precipitated with ammonium sulfate (457 g/liter) and dissolved in 10 mM Tris-chloride (pH 8) containing 1 mM MgCl₂ (and no EDTA) and dialyzed against the same buffer. This preparation was fractionated on a DEAE-cellulose column (1.4 by 11 cm) equilibrated with 10 mM Tris-chloride (pH 8); elution was with 200 ml of buffer containing a linear concentration gradient of MgCl₂ from 0 to 60 mM. The protein in the fractions with the highest specific enzyme activity was recovered by precipitation with ammonium sulfate and was dissolved in, and dialyzed against, 10 mM Tris-chloride (pH 8) containing 1 mM MgCl₂ (no EDTA). This preparation was stable when stored frozen at $-15°C$.

Purification of *E. coli* malate synthase. The cell-free extract was treated with protamine sulfate as described above for the thermophile synthase. The supernatant liquid was fractionated on a DEAE-cellulose column (2.6 by 28 cm) equilibrated with 5 mM Tris-chloride (pH 8) containing 1 mM MgCl₂, using a linear gradient of 1 to 100 mM MgCl₂ in 800 ml of the buffer. Malate synthase type A, the major species produced by wild-type *E. coli* during growth on acetate as carbon source and distinct from the type G enzyme which is predominantly formed during growth on glycolate (9), was eluted with approximately 50 mM MgCl₂. The active fractions were combined and fractionated with ammonium sulfate; the protein precipitated between 50 and 75% saturation levels was spun down and dissolved in 5 mM Tris-chloride (pH

8) containing 1 mM MgCl₂ and dialyzed against this buffer. This preparation was adjusted to pH 7 by addition of 0.1 M KH₂PO₄, diluted with an equal volume of 5 mM potassium phosphate buffer (pH 7), and applied to a hydroxyapatite column (2 by 12 cm) equilibrated with the phosphate buffer. The enzyme was eluted by passing 200 ml of the phosphate buffer increasing linearly in concentration from 5 to 50 mM. The active fractions were pooled, adjusted to pH 8 with 0.1 M Tris, and fractionated on a DEAE-cellulose column (1.4 by 10 cm) equilibrated with 10 mM Tris-chloride (pH 8) containing 1 mM MgCl₂. The column was eluted with 200 ml of Tris-chloride buffer linearly increasing in MgCl₂ concentration from 1 to 60 mM. Fractions having a specific activity better than 30 were combined and concentrated by vacuum membrane filtration using a Millipore Immersible Molecular Separator. The concentrate was dialyzed against 10 mM Tris-chloride (pH 8) containing 1 mM MgCl₂ and stored at 4°C.

RESULTS

Purification of thermophilic *Bacillus* and *E. coli* malate synthases. The course of purification of the thermophile synthase is summarized in Table 1. The preparations isolated from the wild type and mutant PC2 NG35 were identical in final specific activity and in several other properties such as native and subunit molecular weights, electrophoretic behavior, optimum pH for activity, K_m for glyoxylate and for acetyl coenzyme A, and thermostability. This suggests that the mutant produces the same enzyme species as the wild type but at a higher level, which accounts for nearly 5% of the soluble proteins in the cell (Table 1). Therefore, for the studies reported in this paper malate synthase isolated from the mutant was generally used. Upon electrophoresis of the denatured enzyme in SDS-polyacrylamide gel, a single major polypeptide

TABLE 1. *Purification of thermophilic Bacillus malate synthase*[a]

	Wild type				Mutant PC2 NG35			
Step	Protein (mg)	Enzyme activity (U)	Sp act (U/mg of protein)	Yield (%)	Protein (mg)	Enzyme activity (U)	Sp act (U/mg of protein)	Yield (%)
1. Cell-free extract	4,034	2,154	0.53	100	1,634	2,353	1.44	100
2. Protamine sulfate treatment	3,590	2,111	0.59	98	1,556	2,365	1.52	100
3. Ammonium sulfate fractionation: dialyzed 50 to 65% saturation fraction	1,246	1,657	1.33	70	678	1,764	2.6	75
4. Effluent from phosphocellulose column at pH 6	534	1,314	2.46	61	348	1,741	5.0	74
5. DEAE-cellulose chromatography at pH 7.5: fractions from gradient elution with NaCl	25.2	625	24.8	29	45.8	1,176	25.7	50
6. DEAE-cellulose chromatography at pH 8: fractions from gradient elution with MgCl₂	19.2	517	26.9	24	39	1,059	27.0	45

[a] Samples of 70 g (wet weight) of wild-type cells and 25 g (wet weight) of mutant PC2 NG35 cells were used. Enzyme activity was assayed at pH 8 and 30°C in the system containing DTNB.

band was detected (Fig. 1), indicating the homogeneity of the purified preparation. This was confirmed by the appearance of a single, symmetrical peak during high-speed sedimentation velocity runs in the ultracentrifuge, by the linearity of the plot of log absolute fringe number against the square of the radial distance (3) obtained from low-speed sedimentation equilibrium runs and by the detection of a single amino acid, serine, at the amino terminus of the protein (R. M. Chell, T. K. Sundaram, and A. E. Wilk-

inson, manuscript in preparation).

The results of the purification of the *E. coli* malate synthase are outlined in Table 2. The method described by Falmagne and Wiame (9) did not work satisfactorily in our hands. The specific activity of our purified preparation, however, appears to be comparable to that of their final preparation. The gel electrophoretic patterns obtained with the native and denatured enzymes (Fig. 2) indicate the high state of purity of our preparation. From a densitometric scan of gel A in Fig. 2, the purity was estimated to be better than 80%.

Molecular weights and subunit structures of thermophilic *Bacillus* and mesophilic bacterial malate synthases. The native molecular weight of the thermophile synthase, determined by gel filtration of the pure enzyme or of a cell-free extract of the *Bacillus* cells through Sephadex G-200, is 62,000. This value has been confirmed by the results of sedimentation equilibrium studies in the ultracentrifuge by both the conventional low-speed sedimentation and the high-speed meniscus depletion methods (3) (Chell et al., manuscript in preparation). The molecular weight of the polypeptide produced upon denaturation of the thermophile enzyme in SDS-mercaptoethanol (Fig. 1) is 58,000 as estimated from its mobility in SDS-polyacrylamide. This suggests that the thermophile malate synthase is a monomeric protein. Electrophoretic examination of the enzyme after dimethylsuberimidate treatment and denaturation revealed a single polypeptide band,
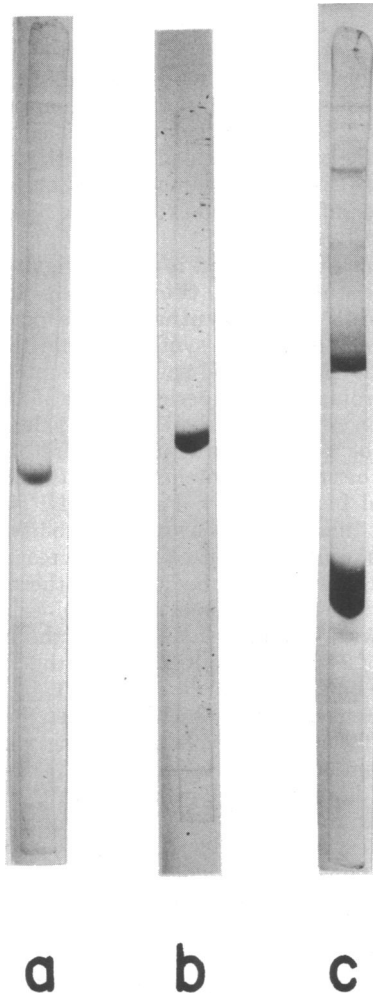

FIG. 1. *SDS-gel electrophoretic profile of denatured thermophilic Bacillus malate synthase. (a) Denatured malate synthase (10 μg); (b) dimethylsuberimidate-treated and denatured malate synthase (15 μg); (c) thermophilic Bacillus isocitrate lyase (2 mg/ml), reacted with dimethylsuberimidate (2 mg/ml) for 72 h at pH 8.5 at room temperature and denatured; protein was subjected to electrophoresis in 15-μg samples.*

TABLE 2. *Purification of E. coli malate synthase[a]*

Step	Protein (mg)	Enzyme activity (U)	Sp act (U/mg of protein)	Yield (%)
1. Cell-free extract	2,983	3,550	1.19	100
2. Protamine sulfate treatment: supernatant fraction	2,623	3,541	1.35	99
3. DEAE-cellulose chromatography: fractions concentrated and dialyzed	392	1,680	4.29	47
4. Ammonium sulfate fractionation: dialyzed 50 to 75% saturation fraction	184	1,439	7.8	40
5. Hydroxyapatite chromatography	27.5	715	26.0	20
6. DEAE-cellulose chromatography: fractions concentrated and dialyzed	9.1	312	34.3	9

[a] A sample of 30 g (wet weight) of cells was used. Enzyme activity was assayed at pH 8 and 30°C in the system without DTNB.
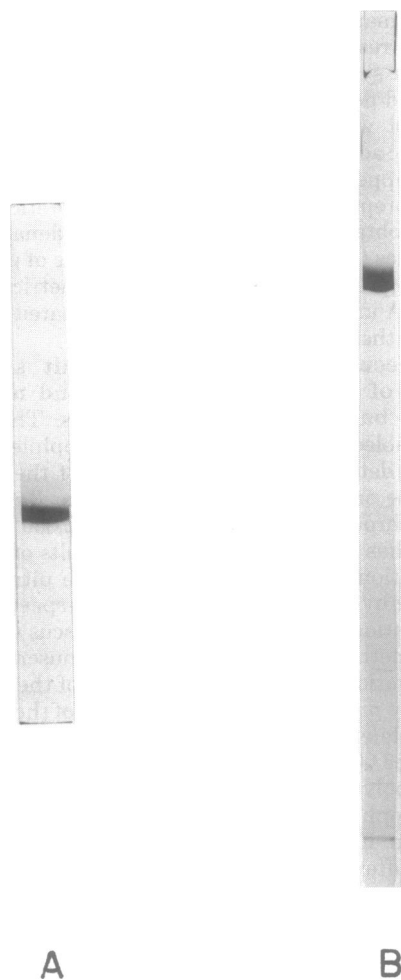
A   B

FIG. 2. *Gel electrophoretic profile of highly purified E. coli malate synthase. (A) Native enzyme (25 µg of protein) subjected to electrophoresis in polyacrylamide and stained for protein; the position of the main protein band corresponded with the position of the band visualized in another gel stained for enzyme activity. (B) Denatured enzyme (10 µg of protein) subjected to electrophoresis in SDS-polyacrylamide and stained for protein.*

which had the same mobility as the band seen after denaturation without the cross-linking treatment. By contrast, isocitrate lyase, the other glyoxylate cycle enzyme, from the thermophilic *Bacillus* yielded multiple bands (Fig. 1), as expected from its multimeric structure (5). This confirms the monomeric nature of the malate synthase.

The native molecular weight of the *E. coli* malate synthase, determined by gel filtration, is 55,000. This value agrees well with that derived from ultracentrifugation experiments (9). When

the highly purified enzyme was denatured in SDS-mercaptoethanol and subjected to electrophoresis in SDS-polyacrylamide, a single major polypeptide was observed (Fig. 2), the mobility of which corresponded to a molecular weight of 56,000. The *E. coli* synthase, like its homolog from the thermophilic *Bacillus*, is thus a monomeric protein. The native molecular weights of malate synthases from *P. indigofera* and *B. licheniformis*, which were deduced from the results of gel filtration of cell-free extracts of these bacteria to be about 56,000, are closely similar to those of the thermophile and *E. coli* enzymes. It is likely therefore that the *P. indigofera* and *B. licheniformis* synthases are also monomers. The values observed here for these two species may be assumed to be reasonable approximations to the true molecular weights, since identical values were obtained for the thermophile synthase when the homogeneous preparation and the unfractionated extract were used in the gel filtration.

Effect of increased ionic strength on the thermostability of the thermophile and mesophile malate synthases. The monomeric nature of the malate synthases from the thermophilic *Bacillus* and several mesophilic bacteria prompted an experimental verification of the suggestion (18) that specific salt bridges might be a unique structural feature of monomeric thermophile proteins, accounting for their unusual heat stability. Perutz and Raidt (18) predict that a protein owing its stability to salt bridges would be labilized by an increase in the ionic strength of the medium. The thermophile malate synthase possesses considerable heat stability with estimated half-lives, in the purified state, of 2.8 h and 12.5 h at 60°C in the Tris and glycine buffer systems described in Table 3. The *E. coli* enzyme in the highly purified state was markedly more thermolabile, having a half-life of 25 min at 45°C and 3 min at 50°C (Table 3); its half-life in cell-free extract at 45°C was also about 25 min (Table 4). Malate synthases from the other two mesophilic bacteria, *P. indigofera* and *B. licheniformis*, which were examined only in cell-free extracts, were also appreciably less resistant to thermal denaturation than the thermophile synthase (Table 4). When the ionic strength was increased by the addition of 0.2 M potassium chloride, a striking labilization of the thermophile enzyme occurred at 60°C to a half-life of about 30 min in the Tris system and of 50 min in the glycine system. Potassium chloride also caused a decrease in thermostability at 65°C. A similar pattern was seen at pH 9 in the glycine system, the half-lives at 60°C without potassium chloride and with 0.2 M potassium chloride being 8.7 h and 12 min, respectively

TABLE 3. *Thermostability of purified malate synthases from the thermophilic Bacillus and E. coli*[a]

Enzyme source	Buffer	Temp (°C)	Half-life (min)	
			Without KCl	With 0.2 M KCl
Thermophilic Bacillus	25 mM Tris-chloride (pH 7)	60	168	30
	25 mM Glycine-NaOH (pH 8.5)	60	750	50
	25 mM Glycine-NaOH (pH 9)	60	522	12
	25 mM Tris-chloride (pH 6.86)	65	6	2
E. coli	50 mM Tris-chloride (pH 8 at 20°C)	45	25	25
	50 mM Tris-chloride (pH 8 at 20°C)	50	3	3

[a] All buffers contained 0.1 mM MgCl₂. The longer half-lives (30 min and over) were estimated from inactivation plots established over 80 to 100 min. The thermophilic *Bacillus* enzyme was heated at a concentration of 16 μg of protein per ml, and the *E. coli* enzyme was heated at a concentration of 30 μg of protein per ml. After the heat treatment the samples were quickly cooled in ice and assayed for enzyme activity, the thermophile enzyme in the system with DTNB, and the *E. coli* enzyme in the system without DTNB. The pH values of the Tris and glycine buffers given for the thermophile system are those estimated for the temperatures of the heat treatment from the pH measured at 20°C and the temperature coefficients (dpH/dt) of −0.028 and −0.026 pH unit/°C, respectively (7).

(Table 3). The effect of salt on the stability of the *E. coli* malate synthase was examined at 45°C and 50°C because of its extreme instability at the higher temperatures. At either temperature, potassium chloride made little difference to the rate of thermal inactivation of this enzyme (Table 3). The response of the *P. indigofera* and *B. licheniformis* synthases to potassium chloride, which was studied in cell-free extracts, was likewise different from that of the thermophile enzyme; the thermophile synthase, although somewhat less unstable in cell-free extract than in the purified state, was still markedly labilized by the salt in the impure system (Table 4).

Since cell-free extracts were used in the study of the stability of the *P. indigofera* and *B. licheniformis* malate synthases, there is the possibility that the results may be subject to complication due to likely protease action in the extracts. However, it is unlikely, but not completely ruled out, on the basis of the following observations, that the conclusion drawn from the above data regarding the effect of salt on the thermostability of the two mesophile enzymes in seriously invalidated by interfering proteolytic activity. The thermophile and *E. coli* syn-

thases were no more stable in the purified state than in cell-free extracts (see above). The thermophile enzyme was completely stable in the extract for at least 2 h at 45°C in both the Tris (pH 8) and glycine (pH 7.5 to 9.5) buffer systems and therefore resistant to protease action under these conditions. The stability of the *P. indigofera* and *B. licheniformis* synthases in cell-free extracts in Tris-chloride buffer (pH 8) at 45°C was unaffected by the inclusion of the protease inhibitor, phenylmethylsulfonyl fluoride (25 μg/ml), in the presence or absence of potassium chloride.

Effect of high pH on the heat stability of thermophilic Bacillus and E. coli malate synthases. A weakening of salt bridges in proteins might be brought about by an increase in pH as well as in ionic strength (18). The thermostability of the thermophile malate synthase was therefore determined at several pH values, as shown in Fig. 3. The enzyme possessed considerable stability at 60°C in the pH range of 7.5 to 9 with half-lives of approximately 4 to 12 h. When the pH was raised above 9, an extremely steep drop in stability resulted, with half-lives of 15 min and 1 min at pH 9.5 and 10, respectively. The *E. coli* enzyme behaved differently, exhibiting a gradual decrease in thermostability at 45°C as the pH was increased from 7.5 to 9.5. As stated above, the thermophile synthase was

TABLE 4. *Thermostability of thermophile and mesophile malate synthases in unfractionated cell-free extracts*[a]

Organism	Temp (°C)	Half-life (min)	
		Without KCl	With 0.2 M KCl
B. licheniformis	40	210	210
	45	25	25
	50	4	1.9
P. indigofera	40	82	82
	45	2	2.4
E. coli	45	25.5	25.5
Thermophilic Bacillus	60	348	72
	65	6.8	3.6

[a] Thermostability was determined in cell-free extracts which had been dialyzed against 10 mM Tris-chloride (pH 8) containing 1 mM MgCl₂ and then diluted 10-fold in 50 mM Tris-chloride (pH 8) with the addition of KCl as indicated. The diluted samples contained 0.5 to 0.7 mg of protein per ml. The half-lives of the *B. licheniformis* and *P. indigofera* enzymes at 40°C were estimated by extrapolation of inactivation plots established over 30 min. The half-life of the thermophile enzyme without KCl at 60°C was estimated by extrapolation of the inactivation plot established over 100 min.
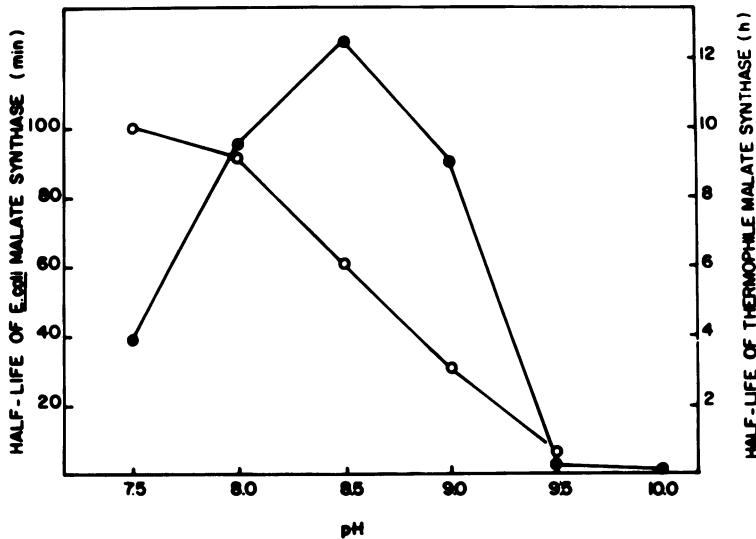
FIG. 3. *Thermostability of thermophilic Bacillus and E. coli malate synthases at different pH values. The stability of the thermophile enzyme (●) at 60°C in 25 mM glycine-sodium hydroxide buffer containing 0.1 mM MgCl₂ at the pH values (estimated at 60°C) indicated was determined. The half-lives were obtained from inactivation plots (extrapolated when necessary) established over periods of up to 100 min. The stability of the E. coli synthase (○) was similarly examined, but at 45°C.*

completely stable at 45°C for at least 2 h in the pH range of 7.5 to 9.5. Its inactivation at 60°C at pH 9.5 without potassium chloride or at pH 9 with 0.2 M potassium chloride was not reversible by dialysis against Tris-chloride buffer (pH 8) containing 1 mM MgCl₂.

DISCUSSION

The only malate synthase for which information is available on the subunit structure is the enzyme from bakers' yeast, which is composed of at least two, and possibly more than two, polypeptide chains (19). The several malate synthases examined in this study from mesophilic and thermophilic bacteria contain but one polypeptide chain; this conclusion is at present somewhat conjectural in the case of the enzymes from *P. indigofera* and *B. licheniformis* and remains to be confirmed by work with purified proteins.

The marked labilization of the thermophile malate synthase by an increase in ionic strength strongly suggests that salt bridges must be a prime thermostabilizing structural component of this monomeric enzyme. The finding that the several mesophilic bacterial malate synthases studied here are not similarly labilized by potassium chloride lends support for this conclusion. The gradual decline in stability at the relatively low temperature of 45°C observed with the *E. coli* enzyme as the pH was raised from 7.5 to 9.5 (Fig. 3) probably represents a somewhat general effect of high pH on proteins. The response of

the thermophile malate synthase at the much higher temperature of 60°C is, however, strikingly characteristic and apparently more specific, especially above pH 9, and is in accord with the general proposal of Perutz and Raidt (18). The isoelectric pH of the thermophile enzyme, determined from its electrophoretic mobilities at various pH values in acetate buffer of constant ionic strength (0.1), is 4.6. The fact that the pH range, 9 to 10, in which extreme labilization of the thermophile synthase occurs is far removed from the isoelectric point indicates that specific salt bridges involving groups having a pK value in the pH range of 9 to 10 may be essential to maintain the structural stability of this enzyme. Elucidation of the amino acid sequence of the synthase and a study of the effect of group-specific reagents on its thermostability may reveal the crucial residues required for the establishment of the specific salt bridges. It has been estimated that a free energy of stabilization of less than 3 kcal (ca. 12.6 kJ) per mol can account for the heat stability of most thermophile proteins (18, 27). This relatively small energy demand could be met in manifold ways, justifying the currently held view that there need not be a single general mechanism of thermostabilization for all thermophile proteins.

LITERATURE CITED

1. **Andrews, P.** 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. **96**:595–606.
2. **Atkinson, A., P. A. Bradford, and I. P. Selmes.** 1973. The large scale preparation of chromatographic grade hydroxyapatite and its application in protein separation procedures. J. Appl. Chem. Biotechnol. **23**:517–529.
3. **Bowen, T. J.** 1970. An introduction to ultracentrifugation. Wiley-Interscience, London.
4. **Cazzulo, J. J., T. K. Sundaram, and H. L. Kornberg.** 1970. Properties and regulation of pyruvate carboxylase from *Bacillus stearothermophilus*. Proc. R. Soc. London Ser. B **176**:1–19.
5. **Chell, R. M., and T. K. Sundaram.** 1975. Isolation and characterization of isocitrate lyase and malate synthase from *Bacillus stearothermophilus*. Biochem. Soc. Trans. **3**:303–306.
6. **Davis, G. E., and G. R. Stark.** 1970. Use of dimethylsuberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins. Proc. Natl. Acad. Sci. U.S.A. **66**:651–656.
7. **Dawson, R. M., D. C. Elliott, W. H. Elliott, and K. M. Jones.** 1972. Data for biochemical research, p. 483–498. Oxford University Press, London.
8. **Dixon, G. H., and H. L. Kornberg.** 1962. Malate synthetase from baker's yeast. Methods Enzymol. **5**:633–637.
9. **Falmagne, P., and J. M. Wiame.** 1973. Purification et caracterisation partielle des deux malate synthases d'*Escherichia coli*. Eur. J. Biochem. **37**:415–424.
10. **Griffiths, M. W., and T. K. Sundaram.** 1973. Isocitrate lyase from a thermophilic *Bacillus*: effect of salts on enzyme activity. J. Bacteriol. **116**:1160–1169.
11. **Kornberg, A.** 1955. Lactic dehydrogenase of muscle. Methods Enzymol. **1**:441–443.
12. **Kornberg, H. L.** 1966. Anaplerotic sequences and their role in metabolism. Essays Biochem. **2**:1–31.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J.**
Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
15. **McFadden, B. A.** 1969. Isocitrate lyase. Methods Enzymol. **13**:163–170.
16. **Ochoa, S.** 1955. Malic dehydrogenase from pig heart. Methods Enzymol. **1**:735–739.
17. **Ornstein, L., and B. J. Davies.** 1961. Disc electrophoresis. Preprint. Kodak Ltd., Kirkby, Liverpool, U.K.
18. **Perutz, M. F., and H. Raidt.** 1975. Stereochemical basis of heat stability in bacterial ferredoxins and in haemoglobin A2. Nature (London) **255**:256–259.
19. **Schmid, G., H. Durchschlag, G. Biedermann, H. Eggerer, and R. Jaenicke.** 1974. Molecular structure of malate synthase and structural changes upon ligand binding to the enzyme. Biochem. Biophys. Res. Commun. **58**:419–426.
20. **Singleton, R., Jr., and R. E. Amelunxen.** 1973. Proteins from thermophilic microorganisms. Bacteriol. Rev. **37**:320–342.
21. **Strecker, H. J.** 1955. L-Glutamic dehydrogenase from liver. Methods Enzymol. **2**:220–225.
22. **Sundaram, T. K.** 1972. Myo-inositol catabolism in *Salmonella typhimurium*: enzyme repression dependent on growth history of organism. J. Gen. Microbiol. **73**:209–219.
23. **Sundaram, T. K.** 1973. Physiological role of pyruvate carboxylase in a thermophilic bacillus. J. Bacteriol. **113**:549–557.
24. **Sundaram, T. K., J. J. Cazzulo, and H. L. Kornberg.** 1969. Anaplerotic CO₂ fixation in mesophilic and thermophilic bacilli. Biochim. Biophys. Acta **192**:355–357.
25. **Warburg, O., and W. Christian.** 1941. Isolierung and Kristallisation des Garungsferments Enolase. Biochem. Z. **310**:384–421.
26. **Weber, K., J. R. Pringle, and M. Osborn.** 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Methods Enzymol. **26**(Part C):3–27.
27. **Wilkinson, S., and J. R. Knowles.** 1974. A comparison of purified valyl-transfer ribonucleic acid synthetase from *Bacillus stearothermophilus* and from *Escherichia coli*. Biochem. J. **139**:391–398.