Tetrameric malate dehydrogenase from a thermophilic *Bacillus*: cloning, sequence and overexpression of the gene encoding the enzyme and isolation and characterization of the recombinant enzyme

Samantha A. WYNNE*† David J. NICHOLLS†, Michael D. SCAWEN† and Trichur K. SUNDARAM*‡

* Department of Biochemistry and Applied Molecular Biology, UMIST, PO Box 88, Manchester M60 1QD, U.K. and † Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts. SP4 0JG, U.K.

The gene encoding the tetrameric malate dehydrogenase (MDH) in a thermophilic *Bacillus* species (BI) has been cloned in an *Escherichia coli* plasmid. The nucleotide sequence of the gene, the first to be elucidated for a tetrameric MDH, shows the MDH subunit to contain 312 amino acids and have a molecular mass of 33648 Da, which confirms the experimentally determined value of about 35 kDa. Like the genomic DNA of BI, the MDH gene is relatively AT-rich; this contrasts with the generally GC-rich nature of the DNA of thermophilic *Bacillus* species. Comparison of amino acid sequences reveals that BI MDH bears greater structural similarity to lactate dehydrogenases (LDHs) than to other (dimeric) MDHs. MDHs and LDHs resemble each other in catalytic mechanism and several other respects. However, whereas MDHs in the majority of organisms are dimers, the tetrameric structure is favoured among LDHs. The stronger structural resemblance that BI MDH has to LDHs than to the dimeric MDHs provides some explanation as to why *Bacillus* MDH, unlike most other MDHs, is tetrameric. A 1 kb fragment containing the BI MDH gene, produced in a PCR, has been cloned into a high-expression *E. coli* plasmid vector. BI MDH synthesized from this clone constitutes about 47% of the total protein in cell extracts of the *E. coli* strain carrying the clone. MDH purified from BI and that purified from the *E. coli* strain carrying the MDH gene clone appear to be identical proteins by several criteria. A number of characteristics of the MDH have been elucidated, including the molecular masses of the native enzyme and the subunit, N-terminal amino acid sequence, isoelectric point, pH optimum for activity, thermostability, stability to pH, urea and guanidinium chloride and several kinetic parameters. Whereas the MDH is a stable tetramer in the pH range 5–7, it appears to be converted into a stable dimer at pH 3.5. This suggests that the dimer is a stable intermediate in the dissociation of the tetramer to monomers at low pH.

INTRODUCTION

Malate dehydrogenase (L-malate–NAD^+ oxidoreductase; EC 1.1.1.37; MDH), an enzyme in the tricarboxylic acid cycle, is a homodimeric molecule in most organisms, including all eukaryotes examined so far, but exists as a homotetramer in some micro-organisms, notably the *Bacillus* species [1,2]. The dimeric MDH has been well studied from several organisms. Thus the gene encoding this MDH species has been cloned and sequenced from *Escherichia coli* [3,4], *Thermus flavus* [5] and *Thermus aquaticus* [6]; X-ray crystallographic studies have yielded three-dimensional structures for the MDHs from porcine heart mitochondria [7] and cytosol [8], *E. coli* [9] and *T. flavus* [10]. By contrast, there is considerably less information about the tetrameric MDHs. No gene for this MDH species has yet been sequenced and none of these enzymes has been studied by X-ray crystallography. An interesting question is whether there are any specific structural features that cause these MDHs to aggregate as tetramers. Immunological comparison of the dimeric and tetrameric MDHs showed that the native tetrameric MDHs from *Bacillus* species, thermophilic and mesophilic, exhibited good cross-reaction but there was no significant cross-reaction between native tetramers and native dimers, suggesting little surface structural similarity between these types of oligomeric MDHs [1,11]. However, significant cross-reaction was observed between the subunits of all MDHs examined, indicating some sequence similarity even between the dimeric and tetrameric enzymes [11].

Lactate dehydrogenase (L-lactate–NAD^+ oxidoreductase; EC 1.1.1.27; LDH) is a 2-hydroxy acid dehydrogenase like MDH. These two enzymes catalyse similar reactions; in both cases there is reversible stereospecific transfer of a hydride ion from a reduced substrate (L-malate or L-lactate) to the oxidized coenzyme (NAD^+) yielding the reduced coenzyme (NADH), a proton and the oxidized substrate (oxaloacetate or pyruvate). The two enzymes also have similar three-dimensional structures [12,13], and several amino acid residues crucial in catalysis are conserved between the two enzymes. However, the level of similarity between the overall primary structures of MDH and LDH is rather low [14]. Eukaryotic LDHs are tetrameric [15]. Bacterial LDHs can form dimers and tetramers, but the tetramer has higher affinity for the substrate and is more active than the dimer and is stabilized by activating compounds such as fructose 1,6-bisphosphate [16].

In the present investigation we have cloned and overexpressed in *E. coli* the gene encoding the tetrameric MDH in a thermophilic *Bacillus*. The cloned gene has been sequenced and the sequence compared with those of several other MDHs and LDHs. We also report the isolation of *Bacillus* MDH from the overproducing *E. coli* strain and several of its characteristics.

MATERIALS AND METHODS

Bacterial strains

The bacterium from which the MDH gene was cloned is a moderately thermophilic *Bacillus* species growing optimally at

Abbreviations used: MDH, malate dehydrogenase; LDH, lactate dehydrogenase; X-Gal, 5-bromo-4-chloro-indolyl β-D-galactoside; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; GdmCl, guanidinium chloride; BI, thermophilic *Bacillus* species; ORF, open reading frame.

‡ To whom correspondence should be addressed at: 13102 Broadmore Road, Silver Spring, MD 20904, U.S.A.

55–58 °C. It was characterized taxonomically as an amylase-negative strain of *Bacillus stearothermophilus* [17]. However, there is some doubt about this classification in view of its DNA base composition. We refer to this strain as BI.

The other bacterial strains used were: *E. coli* TG-1 [*Sup*E, *hsd* 5, *thi*, Δ(*lac-pro* AB), (F′, *tra*D36, *1pro*AB$^+$, *lac*I^q, *lacZ*, ΔM15)]; *E. coli* Top 10 [*mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Ø80, Δ*lac*ΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara*, *leu*) 7679, *gal*U, *gal*K, λ^-, *rps*L(Str), endA1, *nup*G]; *E. coli* DH5α [*Sup*E$_{44}$, Δ*lac* U169 (Ø80 *lacZ*ΔM15), *hsd* R17, *rec*A1, *end*A1, *gyr*A96, *Thi*-1, *rel*-A1].

For the isolation of BI DNA, the bacterium was grown aerobically at 55 °C in DYT broth (1.6 % tryptone/1 % yeast extract/0.5 % NaCl, pH 7.4). For MDH isolation, BI was grown aerobically at 55 °C in continuous culture in a salts medium [18] containing 50 mM sodium acetate as the carbon source, at a dilution rate of 0.25–0.35 h^{-1}. The effluent culture was collected in a vessel cooled at 4 °C, and the cells were harvested by centrifugation at 4 °C and stored frozen until used.

E. coli strains were grown aerobically at 37 °C in DYT broth.

Plasmid and phage vectors

The vectors used were pMTL23 [19], pMTL1003 [20] and M13mp19 [21].

Chemicals and reagents

Restriction endonucleases and T4 DNA ligase were purchased from NBL Gene Sciences (Northumberland, U.K.), components of microbial growth media from Difco Laboratories (West Molesey, Surrey, U.K.), ethanol from Hayman (Witham, Essex, U.K.) and radiolabelled compounds from Dupont U.K. (Stevenage, Herts., U.K.). Other chemicals and reagents were obtained from BDH Chemicals (Poole, Dorset, U.K.), Sigma Chemical Co. (Poole, Dorset, U.K.) and Boehringer-Mannheim (BCL) (Lewes, Sussex, U.K.).

Preparation of BI gene bank

BI chromosomal DNA was partially digested at 37 °C with *Sau*3A restriction enzyme to produce fragments in the 6–7 kb range. The digest was electrophoresed in 0.8 % agarose gel, and the 6–7 kb fragments fraction was isolated from the gel. The plasmid vector pMTL23 was digested with *Bam*HI enzyme at 37 °C and then heated at 70 °C for 10 min. The cut vector was dephosphorylated with calf intestine alkaline phosphatase and purified by phenol extraction and ethanol precipitation.

The BI chromosomal fragments isolated from the electrophoretic gel were ligated at 14 °C into the purified cut vector with T_4 DNA ligase, and the ligation mixture was used to transform *E. coli* Top 10 cells treated with $CaCl_2$ [22]. The transformation mixture was spread on DYT plates containing ampicillin (100 μg/ml), 5-bromo-4-chloro-indolyl β-D-galactoside (X-Gal) (80 μg/ml) and isopropyl β-D-galactoside (IPTG) (50 μg/ml), and the plates were incubated at 37 °C.

Plasmid pMTL23 contains, besides the ampicillin-resistance marker, the *E. coli lac* promoter and *lacZ* gene. White colonies on the DYT–X-Gal plates will putatively carry the plasmid containing the BI DNA inserts within the *lacZ* gene. To confirm this, 20 white colonies were grown in DYT broth containing ampicillin, and plasmid preparations were made from the cultures. The plasmids were digested with *Bam*HI enzyme and electrophoresed in agarose gel; as expected, 6–7 kb DNA fragments were detected in the gel.

More than 4000 white colonies were individually grown in DYT broth supplemented with ampicillin, contained in the wells of a multiwell plate. After the addition of a drop of sterile glycerol to each well, the gene bank was stored at −70 °C.

Isolation of BI chromosomal DNA

DNA was isolated from BI cells (4 g wet weight), harvested from an overnight DYT broth culture, by a modification of the method of Marmur [23], washed sequentially in 70, 80, 90 and 100 % (v/v) ethanol, dried and dissolved in 2 ml of 10 mM Tris/HCl/1 mM EDTA, pH 8, buffer.

Purification of BI MDH

MDH was isolated from acetate-grown BI cells by a modification of the method described previously [24]. MDH from the extract of acetate-grown BI cells, prepared in 40 mM Tris/HCl buffer, pH 7.5, containing 1 mM EDTA, 0.1 mM PMSF and 1 mM dithiothreitol (DTT), was absorbed on a Procion Red HE-3B-Sepharose column. The column was washed with buffer containing 20 mM KCl, and the MDH was eluted with buffer containing 10 mM L-malate and 0.4 mM NAD^+. The MDH was further purified by FPLC on Mono Q (Pharmacia) first at pH 7.2 and then at pH 8.8, the enzyme being eluted by a gradient of 0–1 M NaCl. The buffer at pH 7.2 was 10 mM sodium phosphate/potassium phosphate and that at pH 8.8 10 mM $NaHCO_3$. The MDH in the active fractions from the second anion-exchange chromatography step was precipitated with $(NH_4)_2SO_4$ and stored at 4 °C.

MDH from cells of *E. coli* TG-1 carrying the BIMDH1003 clone was isolated as follows. An extract of the *E. coli* cells grown aerobically overnight at 37 °C in DYT containing ampicillin (100 μg/ml) was heated at 55 °C for 30 min and the supernatant obtained after centrifugation was fractionated on a Procion Red HE-3B-Sepharose column as described above. The active MDH fractions were chromatographed on Mono Q at pH 7.2.

Antibody preparation

MDH purified from BI was diluted to 1 mg/ml in PBS buffer (8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and 1 ml of the diluted enzyme was injected into a rabbit. After 3 weeks, another 0.5 mg of the enzyme was injected. After 6 weeks, 25 ml of blood was drawn from the rabbit and stored overnight at 4 °C. The coagulated blood was centrifuged at 3000 ***g*** for 10 min, and the serum was collected.

Before being used for screening the BI gene bank, the serum was treated with an extract of *E. coli* Top 10 cells in order to neutralize any antibodies to *E. coli* proteins present in the serum. Cells from 6 ml of an *E. coli* Top 10 overnight culture in DYT medium were lysed by the lysozyme/Brij method [24] in a total volume of 400 μl. The serum was incubated with 2 vol. of the lysate at 4 °C for 2 h.

Screening of the BI gene bank for BI MDH gene clones

Clones from the gene bank (4000) were individually inoculated on to Hybond C membranes (Amersham International) placed on DYT agar medium containing ampicillin (100 μg/ml) and grown overnight at 37 °C. The colonies on the membrane were lysed as follows. The membrane was placed in 40 mM Tris/HCl buffer, pH 8, containing 250 μg/ml lysozyme and incubated for 15 min at room temperature. A solution containing 5 % (w/v) Brij 58 and 200 mM $MgSO_4$ was added to the Tris/lysozyme buffer with the membrane to a final concentration of 0.5 % Brij and 20 mM $MgSO_4$, and the incubation was continued for a further 15 min. The membrane was washed for 30 min in PBS

buffer containing Tween 20 (500 μl/l), the cell debris was removed with a sterile glove and the membrane was washed again. The membrane was then agitated in blocking buffer (containing 1 ml of fish skin gelatin per 90 ml of PBS buffer) for 30 min at room temperature. The rabbit antiserum treated with *E. coli* cell lysate was diluted 3000-fold in blocking buffer and the membrane was placed in this and agitated for 2 h, care being taken to prevent the membrane drying and irreversibly binding the antibody. The membrane was then washed three times in PBS/Tween 20 buffer and immersed in the revealing antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, diluted 3000-fold in PBS/Tween buffer). After agitation for 1.5–2 h, the membrane was washed three times in PBS/Tween buffer, and a solution of diaminobenzidine and H_2O_2 (one tablet of diaminobenzidine dissolved in 15 ml of PBS buffer with 12 μl of H_2O_2 added immediately before use) was poured on the washed membrane. Positive colonies turned red within a few minutes.

Control experiments showed that the antiserum was specific for BI MDH and did not interact with *E. coli* MDH and that a 3000-fold dilution of the antiserum revealed at least 1 ng of BI MDH without producing an interfering background on the membrane.

Overexpression of BI MDH gene in *E. coli*

Plasmid BIMDH32 carrying the BI MDH gene was digested with *Bgl*II enzyme, and the 2.3 kb fragment containing the MDH gene was isolated. This fragment was used as the template in a PCR using the primers, 5′-AGGATGGACTCGAGCAT-ATGGCGATTAAAAG-3′ and 5′-GAGCGGTTCTCGAGG-AATTGAAAG-3′. These primers were designed to introduce respectively an *Nde*I site including the translation initiation codon ATG at the start of the MDH-coding sequence and an *Xho*I site approx. 100 bp downstream of the C-terminal codon of the MDH gene. The PCR product, approximately 1 kb long, was ligated into the TA cloning vector (Invitrogen, San Diego, CA, U.S.A.), and the ligation mixture was transformed into the *E. coli* strain supplied with the cloning kit. The transformants contained the BI MDH gene as shown by screening with the anti-MDH serum. The plasmid containing the MDH gene was isolated from a positive transformant, digested with *Nde*I and *Xho*I enzymes, and the 1 kb fragment was isolated. This fragment was ligated into plasmid vector pMTL1003 which had been cut with *Nde*I and *Xho*I enzymes and then treated with calf intestine alkaline phosphatase. pMTL1003 has the *lac* promoter system immediately upstream of the *Nde*I site in the polylinker, and cloning of the 1 kb fragment containing the BI MDH gene into the vector at the *Nde*I site was expected to place the translation initiation codon of the gene at an optimal distance from the ribosome-binding site of this strong promoter system and result in high expression of the MDH gene. The putative recombinant pMTL1003 plasmid carrying the MDH-gene-containing fragment, designated BIMDH1003, was transformed into *E. coli* TG-1. The transformants, appearing white on X-Gal-containing plates as expected, tested positive with anti-(BI MDH) serum, confirming their identity. Restriction analysis of the plasmid isolated from the transformant with *Nde*I and *Xho*I enzymes corroborated this conclusion.

DNA sequencing

The 2.3 kb fragment from BIMDH32 was partially digested with *Sau*3A enzyme to generate 200–600 kb fragments. The fragments were isolated after electrophoresis of the digest in agarose and ligated into the replicative form of M13mp19 phage cut with *Bam*HI enzyme and dephosphorylated with calf intestine alkaline phosphatase. The ligation products were transfected into *E. coli* TG-1. Single-stranded recombinant M13mp19 templates were isolated from the plaques and annealed to universal primer, and DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. [25]. Computer programs supplied by DNASTAR (Madison, WI, U.S.A.) were used to obtain the whole DNA sequence from the sequences of the fragments. Uncertainties in the sequence (on either strand) were resolved by sequencing the plasmid BIMDH32 using oligonucleotide primers designed to sequences 100 bp from the regions of uncertainty. The plasmid used for the sequencing was isolated from *E. coli* DH5α rather than from *E. coli* TG-1 because the former yielded clearer sequences.

The plasmid-sequencing method was also used to verify the sequence of the 1 kb fragment containing BI MDH gene in clone BIMDH1003.

Determination of the level of expression of BI MDH gene from BIMDH1003

The immunotitration method used has been described previously [2]; it takes advantage of the observation that antibodies to BI MDH do not cross-react with *E. coli* MDH. Briefly *E. coli* TG-1 carrying the BIMDH1003 clone was grown aerobically at 37 °C overnight in DYT medium containing ampicillin, and a cell extract was prepared by the lysozyme/Brij method. The cell extract, after suitable dilution, was incubated with various amounts of the anti-(BI MDH) serum, and the BI MDH–antibody complex was precipitated with formalin-fixed *Staphylococcus aureus* cells, which contained Protein A. The supernatant liquid was assayed for MDH activity. The activity removed from the cell extract by the antiserum in this experiment was assumed to be due to BI MDH.

MDH and protein assays

The MDH assay system for oxaloacetate reduction (1 ml) contained 60 mM sodium phosphate/potassium phosphate buffer, pH 7.5, 0.2 mM NADH and 0.3 mM oxaloacetate. The decrease in A_{340} due to the oxidation of NADH was monitored at 30 °C after the addition of enzyme. One unit of enzyme activity was equivalent to the oxidation of 1 μmol of NADH per min. The assay in the direction of malate dehydrogenation was carried out in 0.1 M Tris/HCl buffer, pH 7.5, with 3 mM L-malate, 1 mM NAD^+, 10 mM semicarbazide and enzyme; the rate of increase in A_{340} was measured. Protein was determined by the biuret method [26] in cell extracts and in the 1–10 mg/ml range and by the Bradford method [27] at lower concentrations.

Determination of the N-terminal and internal amino acid sequences of BI MDH

Amino acid sequences were determined using an Applied Biosystems model 477 protein sequencer. About 200 pmol of MDH purified from BI was applied to a poly(vinylidene fluoride) membrane using a Prospin cartridge (Applied Biosystems), and the N-terminal 26 residues were identified. Internal amino acid sequences were obtained as follows. The purified MDH was boiled in 0.1 M Tris/HCl containing 0.1 % (w/v) SDS, pH 8.5, for 5 min and cooled. Sequencing-grade trypsin was added to a final concentration of 2 % (w/w) of the MDH concentration, and the mixture was incubated at 37 °C for 16 h. The proteolysis was stopped by adding anhydrous trifluoroacetic acid to a final concentration of 0.1 %. The peptides were fractionated by

reverse-phase HPLC on a C18 silica column; the elution was carried out with a gradient of methyl cyanide (0–60 %) in 0.1 % trifluoroacetic acid at a flow rate of 1 ml/min. The isolated peptides were applied to polyprene-treated glass-fibre discs for sequence determination.

Recombinant DNA and related methods

Standard techniques [28] were employed.

Molecular mass of BI MDH and its subunit

The molecular mass of the native BI MDH was determined at pH 7 by gel filtration through a 24 ml Superose 12 (Pharmacia) column using an FPLC system; the Superose column was calibrated with marker proteins. The enzyme, contained in 0.2 M Mes buffer, pH 7, supplemented with 0.15 M NaCl, was applied to the column and then eluted with the same buffer mixture at a flow rate of 0.75 ml/min. The molecular mass of the subunit was assessed by SDS/PAGE of the denatured protein on a 10–15 % gradient PhastGel (Pharmacia) alongside marker proteins.

pH optimum for enzyme activity

The rate of oxaloacetate reduction was measured at 30 °C in the pH range 4–10.5, which was covered by four overlapping buffers at 20 mM concentration with 80 mM NaCl added to maintain a high and constant ionic strength. The buffers were: sodium acetate (pH 4.0–5.0), Mes (pH 5.0–7.0), triethanolamine (pH 6.5–8.5) and ethanolamine (pH 8.5–10.5); pH was adjusted with 100 mM HCl or 100 mM NaOH. The concentrations of oxaloacetate and NADH were 0.3 and 0.2 mM respectively. The rate of malate dehydrogenation was measured at 30 °C in the pH range 4.0–12.0. The buffers, at 20 mM concentration with 80 mM NaCl added, were: sodium acetate, Mes, triethanolamine and ethanolamine as above, $NaHCO_3$ (pH 10.5–11.5) and sodium phosphate (pH 11.5–12.0). The malate and NAD^+ concentrations were 3 and 1 mM respectively, and the assay system also contained 10 mM semicarbazide.

Effect of pH on stability of BI MDH

The stability of the MDH was examined over the pH range 4.0–12.0, covered by the buffers used for determining the pH optimum for malate dehydrogenation. Purified MDH, at a concentration of 100 μg/ml, was incubated at room temperature for 30 min in the appropriate buffer, and a portion of this mixture was taken for assay of MDH activity by the standard method (oxaloacetate reduction).

Effect of pH on the quaternary structure of MDH

BI MDH at a concentration of 2 mg/ml was incubated in buffer of the required pH for 30 min at room temperature. The buffers used were sodium acetate (pH 3.5, 4.0, 4.5) and Mes (pH 5.0, 6.0, 7.0) at 200 mM concentration with 150 mM added NaCl. The incubation mixture (100 μl) was loaded on an FPLC Superose 12 gel-filtration column (24 ml) which had been calibrated with marker proteins and equilibrated with the buffer used in the incubation of the MDH. The marker proteins with their molecular masses were: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), *E. coli* MDH (65 kDa) and IgG (150 kDa). MDH was eluted from the Superose column with the same buffer at a flow rate of 0.75 ml/min. The quaternary structure of the enzyme was inferred from the molecular mass estimated from the elution volume.

Stability of BI MDH to guanidinium chloride (GdmCl)

The unfolding of the MDH molecule by GdmCl was monitored by measuring the fluorescence emission at 305 nm after excitation at 278 nm. BI MDH does not contain tryptophan, and therefore the decrease in fluorescence on unfolding was due to the exposure of the tyrosine residues to the solvent. Purified MDH, at a concentration of 70 μg/ml, was incubated in 0.5 M potassium phosphate buffer, pH 7.4, containing 1 mM DTT and GdmCl at the required concentration, for 16 h at 22 °C, and the fluorescence was measured with a Perkin–Elmer LS5 fluorescence spectrophotometer. The fluorescence reading was corrected for the background fluorescence of buffer containing no MDH. The GdmCl concentration was varied in 0.05 M steps in the region of the unfolding transition and in 0.2 M steps outside this region. In preliminary experiments it was established that unfolding equilibrium was attained on 16 h incubation. The unfolding parameters were evaluated by fitting the fluorescence data to the equations given by Staniforth et al. [29] using the non-linear regression-fitting program Enzfitter [30].

Steady-state kinetic parameters

Steady-state initial reaction rates were determined in terms of the rate of change of A_{340} due to the oxidation/reduction of $NADH/NAD^+$, with a Perkin–Elmer Lambda 2 UV/Vis spectrophotometer at 30 °C, with the required concentrations of substrates and coenzymes in the buffer systems used for the standard assays. The data were analysed with Enzfitter, and the kinetic parameters were evaluated.

Electrophoretic techniques

The Phast system (Pharmacia) was used for the electrophoresis of native and denatured proteins according to the manufacturer's instructions. DNA was electrophoresed by the method described by Sambrook et al. [28].

RESULTS

Cloning of BI MDH gene

Screening of the gene bank prepared from BI chromosomal DNA with anti-(BI MDH) serum detected three clear positive clones among 4000 colonies from the bank. Plasmids isolated from the three clones were retransformed into *E. coli* TG-1, and the transformants again tested positive with antiserum. The *E. coli* TG-1 strains carrying the three positive clones were grown in DYT medium containing ampicillin, and cell extracts were prepared and heated at 60 °C for 30 min and assayed for MDH activity. In control experiments, it was observed that this heat-treatment almost completely inactivated *E. coli* MDH and that nearly 42 % of BI MDH activity survived the heat-treatment. These experiments suggested strongly that BI MDH activity was present at significant levels in extracts of cells of the *E. coli* strains that harboured the three clones. These clones, putatively containing the BI MDH gene, were designated BIMDH1, BIMDH2 and BIMDH3.

Restriction analysis of the three recombinant plasmids with several restriction enzymes indicated that they consisted of pMTL23 with BI DNA inserts approx. 6.4–8.8 kb long, that the inserts were similar and that in BIMDH1 the direction of insertion was opposite to that in BIMDH2 and BIMDH3. Since the *lac* promoter in pMTL23 functions unidirectionally, it was also inferred that the BI MDH gene was expressed from its own promoter in at least one of the three clones and hence *E. coli* must be able to recognize the BI promoter.

```
1                                                   50
CACCCAGCTTACATTATGCTTCCGGCTCGTATGTTGTGTGAATTGTGAGC

51                                                  100
GATAACAATTTACACACGAAACAGCTATGACCATGATTACGCCAAGCTTG

101                                                 150
CATGCCTGCAGGTCGACTCTAGAGGATCTATCGATGCATGCCATGGTACC

151                                                 200
CGGGAGCTCGAATTCGGCAATGAATTGATTAAAAACATGGGTTAAACAAA

201                                                 250
GAACTGAGAGCTGCTTCTTTACGAAGTAGCTCTCCATTTAGATTCATTTT

251                                                 300
TGAAAAATCACTTACATAAAAGAGAGGATGGATGCTTGAAATGGCGATTA
                          SD            M  A  I

301                                                 350
AAAGAAAGAAAATTTCTGTCATTGGTGCTGGATTTACTGGTGCGACAACA
A  R  K  K  I  S  V  I  G  A  G  F  T  G  A  T  T

351                                                 400
GCTTTTTTGCTGGCAAAAAAAGAGCTGGGAGATGTTGTGCTCGTTGACAT
 A  F  L  L  A  K  K  E  L  G  D  V  V  L  V  D  I

401                                                 450
TCCGCAAGCAGAGAATCCGACAAAAGGGAAAGCGTTGGACATGCTTGAAT
   P  Q  A  E  N  P  T  K  G  K  A  L  D  M  L  E
451                                                 500
CAAGCCCTGTTCTTGGATTTGATGCGAACATTATCGGAACATCAAACTAT
S  S  P  V  L  G  F  D  A  N  I  I  G  T  S  N  Y

501                                                 550
GAGGAAACGGCTGATTCCGATATTGTTGTGATCACGGCAGGAATTGCAAG
 E  E  T  A  D  S  D  I  V  V  I  T  A  G  I  A  R

551                                                 600
AAAACCTGGAATGAGTCGAGACGATTTAGTGCAGACCAACCAAAAAGTAA
  K  P  G  M  S  R  D  D  L  V  Q  T  N  Q  K  V

601                                                 650
TGAAAAGCGTCACAAAAGAAGTTGTAAAATATTCCCCAAATTCCATTATC
M  K  S  V  T  K  E  V  V  K  Y  S  P  N  S  I  I

651                                                 700
ATTGTGCTGACAAATCCGGTTGATGCAATGACTTACACTGTTTATAAAGA
 I  V  L  T  N  P  V  D  A  M  T  Y  T  V  Y  K  E

701                                                 750
ATCCGGATTTCCAAAACACCGCGTCATCGGTCAATCTGGAGTTTTAGATA
   S  G  F  P  K  H  R  V  I  G  Q  S  G  V  L  D

751                                                 800
CCGCAAGATTCCGCACATTTGTTGCTCAAGAATTGAATTTATCAGTCAAA
T  A  R  F  R  T  F  V  A  Q  E  L  N  L  S  V  K

801                                                 850
GACATTACAGGATTTGTGTTAGGCGGCCACGGAGATGATATGGTGCCATT
 D  I  T  G  F  V  L  G  G  H  G  D  D  M  V  P  L

851                                                 900
AGTTCGCTATTCATACGCAGGGGGCATTCCGTTAGAAAAATTAATCCCGA
  V  R  Y  S  Y  A  G  G  I  P  L  E  K  L  I  P

901                                                 950
AAGAGCGCTTGGAAGCGATTGTTGAAAGAACTAGAAAAGGCGGGGGAGAA
K  E  R  L  E  A  I  V  E  R  T  R  K  G  G  G  E

951                                                1000
ATTGTTAACCTGCTTGGAAACGGAAGCGCATACTACGCACCAGCCGCATC
 I  V  N  L  L  G  N  G  S  A  Y  Y  A  P  A  A  S

1001                                               1050
TCTTGTAGAGATGGTGGAAGCGATTGTTAAAGATCAGCGCCGCGTATTGC
  L  V  E  M  V  E  A  I  V  K  D  Q  R  R  V  L

10051                                              1100
CTGCCATTGCATATTTAGAAGGAGAATACGGTTTTGAAGGTATTTATTTA
P  A  I  A  Y  L  E  G  E  Y  G  F  E  G  I  Y  L

1101                                               1150
GGCGTTCCAACAATTCTTGGAGGCAATGGCTTAGAGCAAATTATTGAGCT
 G  V  P  T  I  L  G  G  N  G  L  E  Q  I  I  E  L

1151                                               1200
TGAACTGACGGATGAAGAAAAAGCAGCTTTGGAAAAATCTGCGGAATCTG
  E  L  T  D  E  E  K  A  A  L  E  K  S  A  E  S

1201                                               1250
TAAGAAATGTTATGAAAGCATTAATATAGTGCATGGTAGAAAAAGGAGGA
V  R  N  V  M  K  A  L  I

1251                                               1300
CATGTTATGTTCCTCCTTTTTGTTATAAAAGAAAAAAGAGAGTTTGCCAT

1301                                               1350
AGAATTCGTCCGCTTTCAATTCCGCTTCAACCGCTCCATCATGTTCTATC

1351                                               1400
GATCTTAAATCTCTGAAGACGCTTCTGATGTCTTCTGCTCGGACATATAT

1401       1413
ATAGATATAGACG
```

Table 1 Codon usage in BI MDH gene

The number of times each codon appears in the coding sequence is shown.

Codon	Amino acid	Number	Codon	Amino acid	Number
GCA	Ala	13	CTG	Leu	5
GCC	Ala	2	CTT	Leu	6
GCG	Ala	7	TTA	Leu	11
GCT	Ala	5	TTG	Leu	6
AGA	Arg	6	AAA	Lys	21
AGG	Arg	0	AAG	Lys	1
CGA	Arg	1			
CGC	Arg	6	ATG	Met	8
CGG	Arg	0			
CGT	Arg	0	TTC	Phe	1
			TTT	Phe	7
AAC	Asn	5			
AAT	Asn	6	CCA	Pro	5
			CCC	Pro	0
GAC	Asp	4	CCG	Pro	5
GAT	Asp	11	CCT	Pro	3
TGC	Cys	0	AGC	Ser	3
TGT	Cys	0	AGT	Ser	1
			TCA	Ser	4
CAA	Gln	5	TCC	Ser	4
CAG	Gln	2	TCG	Ser	0
			TCT	Ser	5
GAA	Glu	18			
GAG	Glu	7	ACA	Thr	9
			ACC	Thr	2
GGA	Gly	15	ACG	Thr	3
GGC	Gly	7	ACT	Thr	4
GGG	Gly	3			
GGT	Gly	5	TGG	Trp	0
CAC	His	2	TAC	Tyr	5
CAT	His	0	TAT	Tyr	6
ATA	Ile	1	GTA	Val	5
ATC	Ile	5	GTC	Val	4
ATT	Ile	19	GTG	Val	7
			GTT	Val	15
CTA	Leu	0			
CTC	Leu	1			

Further analysis, involving digestion with restriction enzymes and screening with anti-(BI MDH) serum, located the MDH gene to a 2.3 kb fragment with a *Bgl*II site at either end. This fragment was isolated from BIMDH3 and cloned into pMTL23, cut with *Bgl*II enzyme, in the right direction for expression of the MDH gene from the vector *lac* promoter. This plasmid clone was designated BIMDH32.

Overexpression of BI MDH gene in *E. coli*

A fragment, approx. 1 kb long, isolated from the PCR system using the 2.3 kb fragment from BIMDH32 as template, was cloned into plasmid pMTL1003. This clone, BIMDH1003,

Figure 1 Nucleotide sequence of BI MDH gene and flanking regions

Sequencing was carried out on the 2.3 kb fragment isolated from the positive clone BIMDH3. A sequence of 1400 nucleotides from this fragment is shown. The putative Shine–Dalgarno sequence (SD) is identified. The amino acid sequence of the MDH, deduced from the nucleotide sequence, is shown below the nucleotide sequence using single-letter abbreviations for the amino acids. The sequences of nucleotides in italics (1–133 and 1296–1413) were determined on only one strand. The N-terminal amino acid sequence and the internal amino acid sequences used to identify the MDH open reading frame are underlined.

Table 2 Amino acid sequence identity, amino acid sequence similarity and structural similarity between BI MDH and other MDHs and LDHs

These comparative data were obtained as explained in the text. Sequence similarity was based on similarity between amino acids as shown in the Tables of Schwartz and Dayhoff [36], and structural similarity was assessed by aligning the α carbon atoms of the protein structures and examining the amino acid substitutions [37].

Enzyme	Sequence identity (%)	Sequence similarity (%)	Structural similarity (%)
B. stearothermophilus LDH	32.2	56.4	80.8
Pig muscle LDH	34.3	54.8	77.9
Pig heart LDH	32.6	53.7	79.8
Dogfish muscle LDH	29.7	52.5	75.9
E. coli MDH	21.5	36.9	70.6
Mouse mitochondrial MDH	21.4	36.5	67.8
Pig mitochondrial MDH	21.4	35.8	67.5
T. aquaticus MDH	16.0	33.9	69.9
Mouse cytosolic MDH	13.3	32.5	67.1
Pig cytosolic MDH	12.7	32.6	67.9

contained the BI MDH gene. The level of BI MDH synthesized in *E. coli* TG-1 cells carrying this clone was determined by immunotitration with anti-(BI MDH) serum. It was inferred that 94% of the MDH activity in the *E. coli* cell extract was due to BI MDH and 6% to *E. coli* MDH. From the specific activity of BI MDH in the cell extract (71 units/mg of protein) and the specific activity of pure BI MDH (150 units/mg of protein) (see Tables 3 and 4), BI MDH accounted for 47% of the protein in the cell extract. A similar value for the level of expression was obtained in an experiment in which MDH activity was assayed after inactivation of the *E. coli* MDH by heat-treatment of the cell extract at 60 °C for 30 min (see above). Using the vector pMTL1003, we have previously achieved a similar level of expression of the *T. aquaticus* MDH gene [31].

The nucleotide sequence of the fragment containing the BI MDH gene in BIMDH1003 was determined on both strands. The sequence of the MDH gene in this fragment was the same as the sequence of the gene in the 2.3 kb fragment carried in BIMDH32, which was derived from the BI gene bank via BIMDH3. This established that the PCR which produced the MDH gene present in BIMDH1003 did not insert any errors into the gene sequence.

Nucleotide sequence of BI MDH gene

A 1400-nucleotide sequence containing the BI MDH-coding region is shown in Figure 1. The MDH-coding sequence, starting at nucleotide 291, was deduced on the basis of the N-terminal amino acid sequence and the amino acid sequences of internal peptides determined with purified BI MDH. This open reading frame (ORF) terminates at nucleotide 1226 and is immediately followed by the translation termination codon TAG. The ORF, 936 nucleotides long, will code for a polypeptide of 312 amino acids with a molecular mass of 33648 Da, which agrees with the experimentally determined value of 35 kDa for the molecular mass of the BI MDH subunit. The isoelectric point of the MDH calculated from the amino acid sequence is 5.23, which compares well with the experimentally determined value of approx. 5.5. An interesting feature of the MDH gene sequence is that it is relatively AT-rich, with an AT mol% value of 58.12. Table 1 presents the pattern of codon usage in the MDH gene. In most of the degenerate third positions of the codons bases A and T

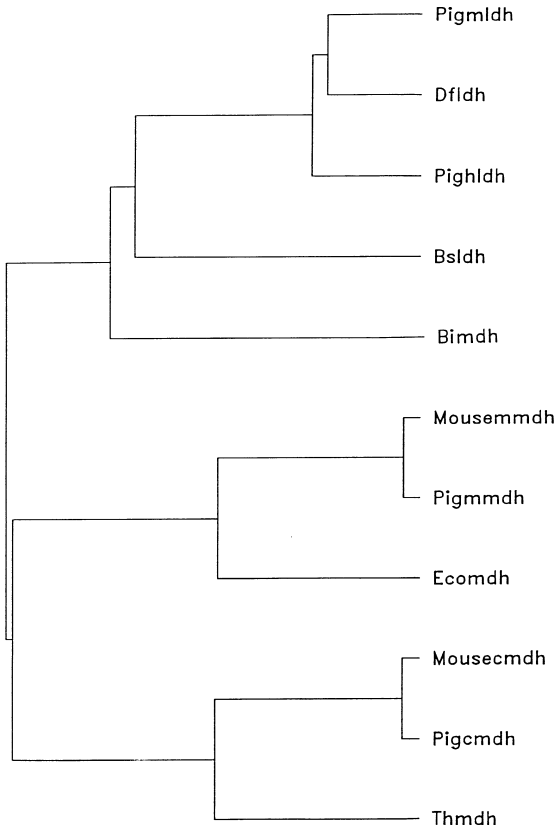


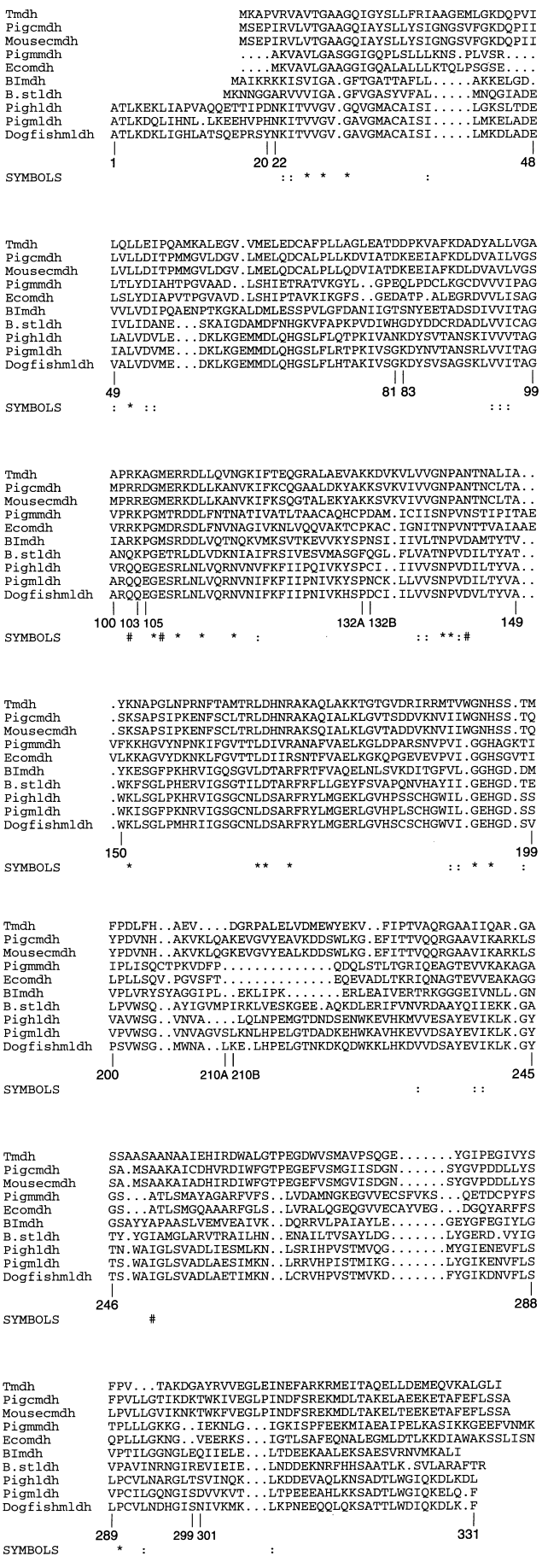
Figure 2 Cluster diagram showing the amino acid sequence similarity of BI MDH to other MDHs and LDHs

The cluster diagram was obtained by using the program PILEUP as explained in the text. Abbreviations are: Pigmldh, pig muscle LDH; Dfldh, dogfish LDH; Pighldh, pig heart LDH; Bsldh, *B. stearothermophilus* LDH; Bimdh, BI MDH; Mousemmdh, mouse mitochondrial MDH; Pigmmdh, pig mitochondrial MDH; Ecomdh, *E. coli* MDH; Mousecmdh, mouse cytosolic MDH; Pigcmdh, pig cytosolic MDH; Thmdh, *T. aquaticus* MDH.

appear more frequently than bases G and C. This pattern is significantly different from that for two other thermophiles belonging to the genus *Bacillus*, namely *B. stearothermophilus* and *Bacillus caldotenax*, the DNA of which is relatively GC-rich [32]. Sequence AGGATG in position 275–280 (Figure 1) is presumably the Shine–Dalgarno sequence, which is thought to have an important role in the initiation of translation [33], as it is quite similar to the canonical Shine–Dalgarno sequence, AAGGAGG, in *Bacillus subtilis* [34].

Amino acid sequence comparison between BI MDH and other MDHs and LDHs

The amino acid sequences of the MDHs and LDHs were compared by alignment using the program PILEUP as implemented in the GCG package of Devereux et al. [35]. The results are shown in Table 2 and Figure 2. From Table 2 a rather surprising conclusion emerges that BI MDH has a higher level of sequence identity, sequence similarity and structural similarity to the LDHs, which are tetramers or more active as tetramers, than to the other MDHs, which are dimers. It is seen from Figure 2 that mitochondrial MDHs cluster with *E. coli* MDH and cytosolic MDHs with *T. aquaticus* MDH. The LDHs cluster separately. BI MDH is situated between the MDH and LDH groups but is more similar to the LDHs than to the MDHs.



Comparison of amino acid residues in specific positions in MDHs and LDHs

The BI MDH amino acid sequence was aligned with the sequences of other MDHs and LDHs in order to compare amino acids in specific positions. The alignment is shown in Figure 3.

BI MDH contains all of the residues conserved in LDHs and other MDHs. These include glycine residues in positions 28, 30 and 33, the only exceptions here being the two cytosolic MDHs, which have alanine in position 33. These three residues in the N-terminal region are part of the nucleotide-binding domain [40]. The residue in position 53 is aspartate in all the enzymes including BI MDH except *T. aquaticus* MDH, in which there is a conservative change to glutamate; this residue is also involved in the binding of the nucleotide coenzyme [40]. A mutant form of *T. aquaticus* MDH carrying aspartate in this position has been produced by Alldread et al. [41]. Arginine-109, aspartate-168, arginine-171 and histidine-195, which are active-site residues [42] conserved in all known MDHs and LDHs, appear in BI MDH.

Some amino acids are conserved differently between MDHs and LDHs. Arginine-102 is totally conserved in MDHs, but LDHs have glutamine in this position. Clarke et al. [43] and Nicholls et al. [44] have shown that the arginine provides substrate specificity for oxaloacetate with MDH and that the glutamine provides specificity for pyruvate with LDH. Methionine-107 and alanine-250 are conserved in the MDHs, and the corresponding conserved residues in LDHs are glutamate and isoleucine. These residues are also probably involved in determining substrate specificity, and BI MDH logically falls in line with MDHs. Arginine-173 is conserved in all LDHs, and a change of this residue to glutamine in *B. stearothermophilus* LDH disrupts the regulation of its tetrameric structure [45]. It is interesting that, of

Table 3 Purification of BI MDH

Acetate-grown BI cells (200 g wet weight) were used.

Purification step	Total enzyme activity (units)	Total protein (mg)	Specific enzyme activity (units/mg of protein)	Yield (%)
Cell extract	3280	8060	0.4	100
Procion Red HE-3B chromatography	2000	30	67	61
FPLC at pH 7.2	1800	17	106	55
FPLC at pH 8.8	920	6	153	28

Figure 3 Alignment of BI MDH amino acid sequence with the sequences of other MDHs and LDHs

The alignment is based on that by Birktoft et al. [38], which took into account structural considerations as well as sequence identity. The numbering of the amino acid residues is based on the numbering system of Eventoff et al. [39] for dogfish muscle LDH. This system was adopted at a time when the exact sequence of this LDH was not known. Consequently in the alignment shown here some numbers are not used (e.g. 21, 82, 104) and sometimes the same number refers to more than one amino acid residue and these residues are distinguished by different letters (e.g. 132A, 132B; 210A, 210B). Symbols: *, totally conserved residue in all the sequences; :, conservative change of a conserved residue; #, totally conserved as one residue in MDHs and as a different residue in LDHs. Abbreviations: Tmdh, *T. aquaticus* MDH; Pigcmdh, pig cytosolic MDH; Mousecmdh, mouse cytosolic MDH; pigmmdh, pig mitochondrial MDH; Ecomdh, *E. coli* MDH; BImdh, BI MDH; B.stldh, *B. stearothermophilus* LDH; Pighldh, pig heart LDH; Pigmldh, pig muscle LDH; Dogfishmldh, dogfish muscle LDH.

Table 4 Purification of BI MDH from *E. coli* cells harbouring clone BIMDH1003

The MDH was purified from *E. coli* TG-1 cells containing a clone of the BI MDH gene in plasmid pMTL1003, grown at 37 °C in a yeast extract/tryptone medium. The cell extract contained both BI MDH and *E. coli* MDH. The heat-treatment inactivated the *E. coli* MDH. Yields of BI MDH are therefore expressed relative to the activity in the heat-treated cell extract.

Enzyme preparation	Enzyme activity (units)	Protein (mg)	Specific enzyme activity (units/mg of protein)	Yield (%)
Cell extract	53826.4	1037.1	51.9	–
Heat-treated cell extract	45420.0	495.1	91.8	100
Eluate from Procion Red column	32718.6	224.1	146	72.1
Eluate from FPLC column	10450.6	69.3	150.9	23.0

Table 5 Effect of pH on the quarternary structure of BI MDH

BI MDH was equilibrated at various pHs and fractionated on an FPLC Superose 12 gel-filtration column at the pH of equilibration. From the elution volume of the MDH from the column, the molecular size (quaternary structure) of the MDH at each pH was estimated. Calibration of the column with marker proteins showed that an elution volume of 13.7 ml corresponded to a molecular mass of 70 kDa (dimer) and an elution volume of 12.7 ml corresponded to a molecular mass of 140 kDa (tetramer).

pH	Elution volume (ml)	Estimate of quaternary structure
7.0	12.70	Tetramer
6.0	12.65	Tetramer
5.0	12.68	Tetramer
4.5	12.90	Tetramer/dimer mixture
4.0	13.21	Tetramer/dimer mixture
3.5	13.71	Dimer

all the MDHs examined here, only BI MDH has this arginine, and this may be an important factor relating to its tetrameric structure. Another residue conserved in LDHs but not in MDHs is aspartate-143 (the corresponding residue is asparagine in MDHs), but BI MDH is LDH-like in this respect.

Purification of BI MDH

The purifications of MDH from BI and *E. coli* TG-1 cells carrying clone BIMDH1003 are summarized in Tables 3 and 4 respectively. The final preparations appeared pure, as shown by the presence of a single protein band on SDS/PAGE of the denatured proteins using a Pharmacia PhastGel system. The two MDH preparations were identical in several characteristics: specific activity, electrophoretic mobility of the native and denatured forms in polyacrylamide, isoelectric point and N-terminal amino acid sequence (see below). We infer therefore that the same MDH is produced in BI and from clone BIMDH1003 in *E. coli*. Characterization of the MDH was undertaken with the purified preparation obtained from *E. coli*, except where indicated otherwise.

Molecular mass of native MDH and subunit

The molecular mass of the native enzyme, determined by gel filtration, was approx. 140 kDa. The molecular mass of the MDH subunit, obtained by SDS/PAGE of the denatured enzyme, was 35 kDa. Thus, as reported previously, the MDH is a stable tetramer.

Other characteristics

The isoelectric point of the MDH, determined by electrophoresis on a narrow range (pH 4.5–6.5) Pharmacia PhastGel with marker proteins, was approx. 5.5; a single protein band was seen from the purified MDH. The value predicted from the nucleotide sequence of the MDH gene is 5.23.

The sequence of the first 15 N-terminal amino acids, determined with the purified enzyme using an Applied Biosystems Protein Sequencer, was AIKRKKIXVIGAGFT, where the residue X could not be unambiguously identified. The same sequence was found in the MDH isolated from BI. The nucleotide sequence of the MDH gene shows that translation is initiated with *N*-formylmethionine. However, the enzymes isolated from BI and the overexpressing *E. coli* both have alanine at the N-terminus. It seems that *E. coli* processes the protein in the same way as BI and removes the N-terminal formylmethionine. It is known that, when alanine is the amino acid following *N*-formylmethionine, it becomes the N-terminal residue in the mature protein [46].

The optimum pH for the reduction of oxaloacetate was 7.5–8.0 at 30 °C and that for the oxidation of L-malate 11–11.5. A higher pH for the oxidation reaction is generally seen with NAD^+-linked dehydrogenases.

Stability of BI MDH activity at different pH values

During incubation for 30 min at room temperature, the enzyme was most stable between pH 7 and 8, where a loss of less than 5% activity was observed. On either side of this pH range, it was appreciably less stable, losing about 20% activity at pH 6 and 9.

Change in BI MDH quaternary structure with pH

This was investigated by gel filtration of the MDH at different pHs through a Superose 12 (Pharmacia) column with an FPLC system. The molecular mass of the enzyme was estimated from its elution volume and the elution volumes of appropriate protein markers from the column. Over the pH range 5–7, the elution volume of the MDH corresponded to a molecular mass of about 140 kDa and a tetrameric structure. As the pH was lowered below 5, the elution volume increased indicating a dissociation of the tetramer. At pH 3.5 the elution volume was indicative of a molecular mass of about 70 kDa and a dimeric structure (Table 5). Since the protein became insoluble below pH 3.5, it was not possible to determine whether monomers would be formed at lower pH. The dissociation of the tetramer to the dimer was reversed when the protein was re-equilibrated at pH 7, as shown by the elution volume from the Superose column and the regain of enzyme activity. It was not possible to establish whether the dimer has any enzyme activity since the activity assay cannot be carried out at the low pH at which the dimer exists in a stable form.

It is evident from these results that BI MDH can exist as a stable dimer at low pH and that the tetramer does not dissociate into monomers in a single step as the pH is lowered. Whereas eukaryotic LDHs are tetramers, bacterial LDHs can exist as dimers and tetramers, the tetrameric form having higher catalytic activity and affinity for the substrate and being stabilized by activators [15,16]. BI MDH, a bacterial MDH structurally more

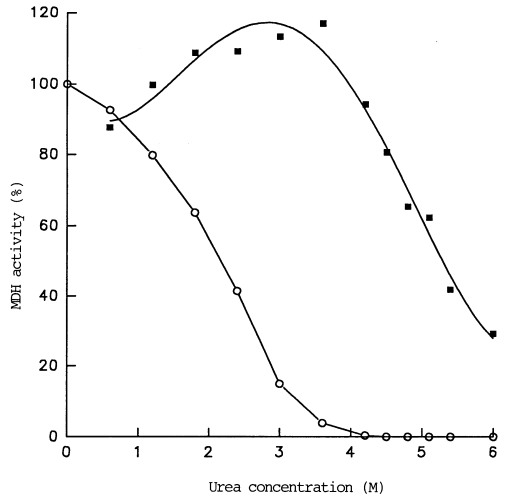


Figure 4 Stability of BI MDH and *E. coli* MDH to urea

MDHs isolated from BI and *E. coli* [4] (0.4 mg/ml) were incubated at 30 °C for 6 min in 60 mM sodium phosphate/potassium phosphate buffer, pH 7.5, containing 0.2 mM NADH with various concentrations of urea, and the enzyme activity was then assayed after the addition of 0.3 mM oxaloacetate. ■, BI MDH; ○, *E. coli* MDH.

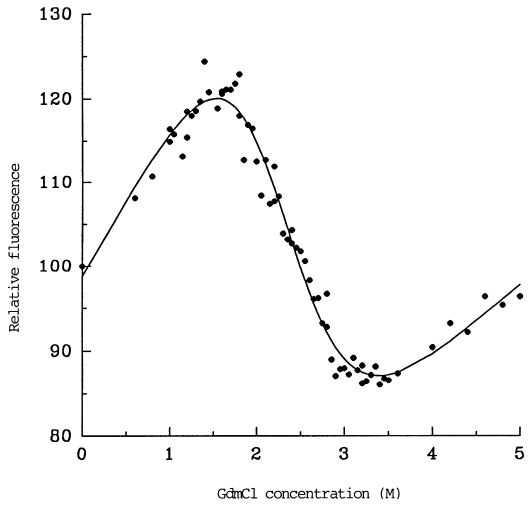


Figure 5 Unfolding of BI MDH by GdmCl

Purified BI MDH (70 μg/ml) was incubated at 22 °C for 16 h in 0.5 M potassium phosphate buffer, pH 7.4, containing 1 mM DTT with various concentrations of GdmCl, and the fluorescence of the incubation mixtures was determined at 305 nm after excitation at 278 nm.

similar to LDHs than to dimeric MDHs, however, has a stable tetrameric structure at physiological pH. The subunit interactions in the tetrameric and dimeric forms of BI MDH would be an interesting subject for investigation.

Thermostability and stability to chemical denaturants

MDH isolated from BI was incubated at 60 °C in 10 mM sodium phosphate/potassium phosphate buffer, pH 7.2, at a protein concentration of 0.4 mg/ml for various periods of time, cooled on ice for 20 min and assayed for enzyme activity at 30 °C. The MDH retained more than 50 % of its activity after incubation at 60 °C for 1 h. In a similar experiment, MDH purified from *E. coli* retained less than 1 % of its activity.

The effect of urea on the two enzymes was studied by incubating MDH isolated from BI and *E. coli* with various concentrations of urea in 60 mM sodium phosphate/potassium phosphate buffer, pH 7.5, containing 0.2 mM NADH at 30 °C for 6 min and then measuring the activity in the presence of urea after the addition of the substrate oxaloacetate (Figure 4). Initially there was an activation of BI MDH, which reached a maximum of about 20 %, as the urea concentration was increased to 3.5 M. Enzyme inactivation occurred at higher concentrations of urea, 50 % of the activity being lost with 5 M urea. *E. coli* MDH showed no initial activation but was steadily inactivated as the urea concentration was raised. The enzyme lost 50 % of its activity at 2 M urea and almost all the activity at 4 M urea.

The unfolding of BI MDH in GdmCl was studied by measuring the fluorescence of the enzyme at various concentrations of GdmCl (Figure 5). There was an initial increase in fluorescence between 0 and 1.5 M GdmCl, possibly caused by the binding of guanidinium ions to the protein. There was then a simple two-state unfolding transition between 1.6 and 3.6 M GdmCl, indicating that subunit dissociation and unfolding occurred simultaneously. The mid-point of the unfolding transition was at 2.27 M GdmCl. The value for ΔG^{w} (see Table 6 for definition) was -21.9 kJ·mol^{-1}, and about 22 internal side chains became solvated during the unfolding process, which is a measure of the co-operativity of the unfolding process.

Table 6 Stability characteristics of BI MDH and three other MDH enzymes

The values for pig cytosolic and *T. aquaticus* MDHs are based on the data of Duffield et al. [47] and those for *E. coli* on the data of Goward et al. [48]. The data were recalculated by the method of Staniforth et al. [29]. The errors are the standard errors derived from the curve-fitting algorithm. ΔG^{w}, conformational stability (free energy of unfolding) in the absence of denaturant; n, number of amino acid side chains solvated during unfolding; $[GdmCl]_{1/2}$, GdmCl concentration at the mid-point of the unfolding transition; $t_{1/2}$, half-life of the MDH for irreversible thermal inactivation at the indicated temperature. *T. aquaticus* MDH did not lose activity at 60 °C for at least 2 h, and the $t_{1/2}$ of this enzyme at 90 °C is shown.

Enzyme source	ΔG^{w} (kJ·mol^{-1})	n	$[GdmCl]_{1/2}$ (M)	$t_{1/2}$ (min)
Pig cytosol	-21.3 ± 2.9	73.0 ± 8.7	0.55	1.8 (60 °C)
E. coli	-61.6 ± 5.4	99.6 ± 8.5	1.27	4.0 (60 °C)
BI	-21.9 ± 1.7	22.4 ± 1.9	2.28	60 (60 °C)
T. aquaticus	-38.9 ± 2.5	32.2 ± 2.1	3.20	34 (90 °C)

Table 6 compares the stability characteristics of the tetrameric BI MDH with three other MDHs, which are dimeric. The BI enzyme is considerably more stable than the two mesophilic enzymes from *E. coli* and pig cytosol, in terms of both denaturation by GdmCl and thermal inactivation. The GdmCl concentrations at the mid-points of the unfolding transitions increase in parallel with the thermal stabilities. The two mesophilic enzymes show a much higher degree of co-operativity of unfolding than the two thermophilic enzymes. The interpretation of the conformational free energies is less clear, as the *E coli* enzyme has a high value, despite being relatively unstable compared with the two thermophilic enzymes. However, conformational free energies must always be considered in conjunction with the co-operativity of the unfolding process.

Table 7 Kinetic parameters of BI MDH

k_{cat} and K_m values (apparent) for each substrate/coenzyme were obtained from initial reaction rates at various concentrations of the substrate/coenzyme at 30 °C with the concentration of the coenzyme/substrate kept fixed. The coenzyme was NADH with the substrates, oxaloacetate, pyruvate and oxoglutarate, and NAD^+ with L-malate. To determine the values for oxaloacetate, pyruvate and oxoglutarate, the NADH concentration was fixed at 0.2 mM; to determine the values for NADH and NADPH, the oxaloacetate concentration was fixed at 0.3 mM; to determine the values for L-malate, the NAD^+ concentration was fixed at 1.0 mM; to determine the values for NAD^+, the L-malate concentration was fixed at 3.0 mM. k_{cat} and K_m values for pyruvate were difficult to determine as the reaction rates were very low. The slope of the initial part of the reaction rate versus [pyruvate] plot was taken to be an estimate of k_{cat}/K_m for this substrate. Errors are S.E.M.s obtained from the Enzfitter program.

Substrate/ coenzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
Oxaloacetate	69.7 ± 11	22 ± 2	3.2×10^6
L-Malate	10.6 ± 2.9	260 ± 60	4.2×10^4
NADH	78.7 ± 11	14 ± 2	5.6×10^6
NADPH	9.4 ± 0.4	130 ± 15	7.2×10^4
NAD^+	11.5 ± 1.5	100 ± 20	1.1×10^5
Pyruvate	–	–	3.4×10^{-1}
α-Oxoglutarate	0.048 ± 0.01	$59\,000 \pm 30\,000$	8.3×10^{-1}

The correlation between thermostability and stability to the chemical denaturant GdmCl that is evident here is frequently, but not always, seen with proteins, although the mechanisms of thermal and chemical denaturation are not the same. The correlation may be related to general protein structural stability.

The present study shows that the thermophilic BI MDH is, as expected, more thermostable and more stable to chemical denaturants than the mesophilic *E. coli* MDH. A proper comparison should be between BI MDH, a tetramer, and a tetrameric MDH from a mesophile. Our previous studies have, however, shown that the tetrameric MDH from the mesophile, *B. subtilis*, is rather less stable to heat and chemical perturbants than *E. coli* MDH [11].

Kinetic characteristics of BI MDH

In Table 7 are given the steady-state kinetic parameters for BI MDH with respect to various substrates and coenzymes. The two k_{cat} values for oxaloacetate reduction by NADH, one derived by varying the oxaloacetate concentration and the other by varying the NADH concentration, are nearly equal as expected, as are the two k_{cat} values for malate oxidation by NAD^+. The rate of oxaloacetate reduction is considerably faster than the rate of malate oxidation at pH 7.5, as is generally the case with NAD^+-linked dehydrogenations. The K_m value for the reduced coenzyme, NADH, is appreciably lower than that for the oxidized coenzyme, NAD^+. This is also observed with NAD^+-dependent dehydrogenases and is probably due to the positive charge on the nicotinamide ring in NAD^+ making the binding of this coenzyme form to the hydrophobic binding pocket in the enzyme energetically less favourable than the binding of the neutral NADH [13].

The specificity of BI MDH for NADH is not absolute as shown by the comparative k_{cat}, K_m and k_{cat}/K_m values for NADH and NADPH (Table 7), although NADH is much the preferred coenzyme. However, pyruvate and α-oxoglutarate substitute for oxaloacetate much less efficiently (Table 7). Although the LDH substrate pyruvate is a poor substrate relative to oxaloacetate for BI MDH, the k_{cat}/K_m value for pyruvate in this system is nearly twice the corresponding parameter for *E. coli* MDH [43]. A possible explanation for this is that structurally the tetrameric BI MDH is more similar to LDHs than to the dimeric MDHs such as *E. coli* MDH.

Citrate, which inhibits malate oxidation and activates oxaloacetate reduction by porcine mitochondrial and cytosolic MDHs and inhibits the binding of malate to *E. coli* MDH, has no effect on the activity of BI MDH. This appears to be another characteristic in which the tetrameric BI enzyme differs from its dimeric counterparts. Like other MDHs, BI MDH is inhibited by the substrate oxaloacetate at higher concentrations.

DISCUSSION

BI MDH has previously been shown [1] to be a tetramer with a subunit molecular mass of about 35 kDa. The subunit molecular mass has been confirmed in this study from the amino acid sequence of the enzyme and also by SDS/PAGE of the denatured MDH. The molecular mass of the native enzyme has also been confirmed to be about 140 kDa by gel filtration of the native MDH. To our knowledge the gene for no other tetrameric MDH has yet been unambiguously identified and sequenced or hyperexpressed. In a recent paper Jin and Sonenshein [49] describe the cloning and sequencing of two citrate synthase genes from *B. subtilis*. They have also sequenced an ORF downstream of one of the citrate synthase genes (*citZ*) and inferred it to be the gene encoding isocitrate dehydrogenase on the basis of sequence similarity to the isocitrate dehydrogenase genes in *E. coli* and *Saccharomyces cerevisiae*, of loss of enzyme activity due to mutation in the gene and of complementation tests. Another ORF, downstream from the isocitrate dehydrogenase gene, which was not sequenced completely, has been tentatively assumed to be the gene for MDH. The *B. subtilis* MDH is also tetrameric [1]. We have recently cloned and sequenced the isocitrate dehydrogenase gene from BI and found it to be just upstream of the MDH gene (R. Dajani, D. J. Nicholls, M. D. Scawen and T. K. Sundaram, unpublished work). Genetic mapping locates the isocitrate dehydrogenase gene and the MDH gene both at 259° on the *B. subtilis* chromosome [50]. Thus in both *B. subtilis* and BI these two genes are located contiguously on the bacterial chromosome, which contrasts with their marked physical separation on the *E. coli* chromosome [51]. It is possible, but not established, that the two genes may be parts of a single operon in the *Bacillus* species.

Comparison of the amino acid sequence of BI MDH with the sequences of LDHs and other (dimeric) MDHs shows that, while BI MDH has all the residues conserved in both MDHs and LDHs, it carries some residues that are conserved only in the MDHs and some conserved only in the LDHs. Amino acids conserved only in the MDHs generally have a function in catalysis, and at least one residue, arginine-173, conserved in LDHs and BI MDH, appears to have a quaternary structural role as indicated by the work referred to above [45] and by more recent work in which the tetrameric LDH of *B. stearothermophilus* was converted into a stable dimeric form by protein engineering [52,53]. By more than one criterion examined in this study, BI MDH exhibits a stronger structural resemblance to the tetrameric LDHs than to the dimeric MDHs. This provides some explanation as to why this MDH, unlike the MDHs from the majority of organisms, is tetrameric. It would be interesting to generate mutant forms of BI MDH, particularly ones in which arginine-173 and aspartate-143 were changed to other amino acids, and examine their quaternary structures. It may be possible to produce a stable dimeric BI MDH in this way, and it would then be pertinent to ask whether such a dimer is enzymically active.

From an amino acid sequence alignment, such as that presented in Figure 3, the mammalian LDHs, which are always tetrameric, are seen to have an N-terminal extension of more than ten amino acid residues in comparison with their bacterial counterparts (in Figure 3 *B. stearothermophilus* LDH is the only bacterial LDH). Bacterial LDHs can exist in either the tetrameric or dimeric form, the tetramer having higher enzyme activity and affinity for the substrate. The N-terminal extension in mammalian LDHs wraps around the protein like an arm and facilitates the aggregation of two dimers to form a stable tetramer [15]. BI MDH does not possess the N-terminal extension but nevertheless exists as a stable tetramer. The detailed molecular basis of the tetrameric structure of *Bacillus* MDH remains to be elucidated.

Soon after its isolation, BI was characterized as an amylase-negative strain of *B. stearothermophilus* by taxonomic tests [17]. From DNA melting data the mol% of GC in BI DNA was worked out to be 47.5% by Sharp et al. [32] and to be 36% by White et al. [54]. Despite this discrepancy, it is evident that BI DNA is relatively AT-rich in contrast with the DNAs of several *B. stearothermophilus* strains and of other thermophiles belonging to the genus *Bacillus*, such as *B. caldotenax*, *Bacillus caldolyticus* and *Bacillus caldovelox*, which all have a GC content of more than 50% [32]. The AT-richness of BI DNA is reflected in the base composition of the BI MDH gene (58.12% AT). If BI is indeed a strain of *B. stearothermophilus*, it is somewhat atypical in the base composition of its genome.

We thank Professor Tony Atkinson for advice and encouragement, Biotechnology & Biological Sciences Research Council, U.K. for a studentship awarded to S.A.W. and Dr. G. Keen and R. Hartwell for the determination of amino acid sequences.

REFERENCES

1 Sundaram, T. K., Wright, I. P. and Wilkinson, A. E. (1980) Biochemistry **19**, 2017–2022
2 Smith, K., Sundaram, T. K. and Kernick, M. (1984). J. Bacteriol. **157**, 684–687
3 Vogel, R. F., Entain, K. D. and Mecke, D. (1987) Arch. Microbiol. **149**, 36–42
4 Nicholls, D. J., Minton, N. P., Atkinson, T. and Sundaram, T. K. (1989) Appl. Microbiol. Biotechnol. **31**, 376–382
5 Nishiyama, M., Matsubara, N., Yamamoto, K., Iijima, S., Uozumi, T. and Beppu, T. (1986) J. Biol. Chem. **261**, 14178–14183
6 Nicholls, D. J., Sundaram, T. K., Atkinson, T. and Minton, N. P. (1990) FEMS Microbiol. Lett. **70**, 7–14.
7 Gleason, W. B., Fu, Z., Birktoft, J. and Banaszak, L. (1994) Biochemistry **33**, 2078–2088
8 Birktoft, J. J., Bradshaw, R. A. and Banaszak, L. J. (1987) Biochemistry **26**, 2722–2734
9 Hall, M. D., Levitt, D. G. and Banaszak, L. J. (1992) J. Mol. Biol. **226**, 867–882
10 Kelly, C. A., Nishiyama, M., Ohnishi, Y., Beppu, T. and Birktoft, J. J. (1993) Biochemistry **32**, 3913–3922
11 Smith, K. and Sundaram, T. K. (1988) Biochim. Biophys. Acta **955**, 203–213
12 Banaszak, L. J. and Bradshaw, R. A. (1975) in The Enzymes (Boyer, P. D., ed.), pp. 369–396, Academic Press, New York
13 Holbrook, J. J., Liljas, A., Steindel, S. J. and Rossmann, M. G. (1975) in The Enzymes (Boyer, P. D., ed.), pp. 191–292, Academic Press, New York
14 McAlister-Henn, L. (1988) Trends Biochem. Sci. **13**, 178–181
15 Grau, U. M., Trommer, W. E. and Rossmann, M. G. (1981) J. Mol. Biol. **151**, 289–307
16 Clark, A. R., Atkinson, T., Campbell, J. W. and Holbrook, J. J. (1985) Biochim. Biophys. Acta **829**, 387–396
17 Epstein, I. and Grossowicz, N. (1969) J. Bacteriol. **99**, 414–417
18 Sundaram, T. K., Cazzulo, J. J. and Kornberg, H. L. (1969) Biochim. Biophys. Acta **192**, 355–357
19 Chambers, S. P., Prior, S. E., Barstow, D. A. and Minton, N. P. (1988) Gene **68**, 139–149
20 Brehm, J. K., Chambers, S. P., Brown, K. J., Atkinson, T. and Minton, N. P. (1991) Appl. Microbiol. Biotechnol. **36**, 358–363
21 Messing, J. and Vieira, J. (1982) Gene **19**, 269–276
22 Cohen, S. N., Chang, A. C. and Hsu, L. (1972) Proc. Natl. Acad. Sci. U.S.A. **69**, 2110–2114
23 Marmur, J. (1961) J. Mol. Biol. **3**, 208–218
24 Smith, K., Sundaram, T. K., Kernick, M. and Wilkinson, A. E. (1982) Biochim. Biophys. Acta **708**, 17–25
25 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463—5467
26 Gornall, A. G., Baradawill, C. J. and David, M. M. (1949) J. Biol. Chem. **177**, 751–766
27 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
28 Sambrook, T., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
29 Staniforth, R. A., Burston, S. G., Smith, C. J., Jackson, G. S., Badcoe, I. G., Atkinson, T., Holbrook, J. J. and Clarke, A. R. (1993) Biochemistry **32**, 3842–3851
30 Leatherbarrow, R. J. (1987) A Non-linear Regression Data Analysis Program for the IBM PC, Elsevier BIOSOFT, Cambridge
31 Alldread, R. M., Nicholls, D. J., Sundaram, T. K., Scawen, M. D. and Atkinson, T. (1992) Gene **114**, 139–143
32 Sharp, R. J., Brown, K. J. and Atkinson, A. (1980) J Gen. Microbiol. **117**, 201–210
33 Shine, J. and Dalgarno, L. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 1342–1346
34 Vellanoweth, R. L. (1993) in *Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics (Sonenshein, A. L., Hoch, J. A. and Losick, R., eds.), pp. 699–711, American Society for Microbiology, Washington, D.C.
35 Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. **12**, 387–395
36 Schwartz, R. M. and Dayhoff, M. O. (1979) in Atlas of Protein Sequences and Structure 5, Suppl. 3 (Dayhoff, M. O., ed.)., pp. 353–358, National Biomedical Research Foundation, Washington, D.C.
37 Risler, J. L., Delorme, M. O., Delacroix, H. and Henaut, A. (1988) J. Mol. Biol. **204**, 1019–1029
38 Birktoft, J. J., Fernley, R. T., Bradshaw, R. A. and Banaszak, L. J. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 6166–6170
39 Eventoff, W., Rossmann, M. G., Taylor, S. S., Torff, H. J., Meyer, H., Keil, W. and Kiltz, H. H. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2677–2681
40 Birktoft, J. J. and Banaszak, L. J. (1984) Pept. Protein Rev. **4**, 1–46
41 Alldread, R. M., Halsall, D. M., Clarke, A. R., Sundaram, T. K., Atkinson, T., Scawen, M. D. and Nicholls, D. J. (1995) Biochem. J. **305**, 539–548
42 Clarke, A. R., Atkinson, T. and Holbrook, J. J. (1989) Trends Biochem. Sci. **14**, 101–105
43 Clarke, A. R., Smith, C. R., Hart, K. W., Wilks, H. M., Chia, W. N., Lee, T. J., Birktoft, J. L., Banaszak, L. J., Barstow, D. A., Atkinson, T. and Holbrook, J. J. (1987) Biochem. Biophys. Res. Commun. **148**, 15–23
44 Nicholls, D. J., Miller, J., Scawen, M. D., Clarke, A. R., Holbrook, J. J., Atkinson, T. and Goward, C. R. (1992) Biochem. Biophys. Res. Commun. **189**, 1057–1062
45 Clarke, A. R., Wigley, D. B., Barstow, D. A., Chia, W. N., Atkinson, T. and Holbrook, J. J. (1987) Biochim. Biophys. Acta **913**, 72–80
46 Ben-Bassat, A. and Bauer, K. (1987) Nature (London) **326**, 315
47 Duffield, M. L., Nicholls, D. J., Atkinson, T. and Scawen, M. D. (1994) J. Mol. Graphics **12**, 14–21
48 Goward, C. R., Miller, J., Nicholls, D. J., Irons, L. I., Scawen, M. D., O'Brian, R. and Chowdhry, B. Z. (1994) Eur. J. Biochem. **224**, 249–255
49 Jin, S. and Sonenshein, A. L. (1994) J. Bacteriol. **176**, 4669–4679
50 Anagostopoulos, C., Piggot, P. J. and Hoch, J. A. (1993) in *Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics (Sonenshein, A. L., Hoch, J. A. and Losick, R., eds.), pp. 425–461, American Society for Microbiology, Washington, D.C.
51 Bachmann, B. J. (1990) Microbiol. Rev. **54**, 130–197
52 Jackson, R. M., Gelpi, J. L., Cortes, A., Emery, D. L., Wilks, H. M., Moreton, K. M., Halsall, D. J., Sleigh, R. N., Behanmartin, M., Jones, G. R., Clarke, A. R. and Holbrook, J. J. (1992) Biochemistry **31**, 8307–8314
53 Cameron, A. D., Roper, D. I., Moreton, K. M., Muirhead, H., Holbrook, J. J. and Wigley, D. B. (1994) J. Mol. Biol. **238**, 615–625
54 White, D., Sharp, R. J. and Priest, F. G. (1993) Antonie van Leeuwenhoek J. Microbiol. **64**, 357–386

Received 6 February 1996; accepted 5 March 1996