Malate Dehydrogenase from Thermophilic and Mesophilic Bacteria. Molecular Size, Subunit Structure, Amino Acid Composition, Immunochemical Homology, and Catalytic Activity†

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ABSTRACT: Malate dehydrogenases isolated from a number of mesophilic, moderately thermophilic, and extremely thermophilic bacteria yield upon denaturation subunits of molecular weight 32 000–36 000. Determination of their native molecular weights shows that some of the enzymes are dimeric and others are tetrameric; the two types are distributed in each of the three classes of bacteria. The amino acid compositions of the enzymes show no consistent trend that can be related to the progression of thermostability from the mesophile through the moderate thermophile to the extreme thermophile species. The tetrameric enzyme species all exhibit a high level of structural homology as judged by the criterion of immunological cross-reaction. Little cross-reaction occurs, however, between the tetramers and the dimers. The dimeric enzyme from the extreme thermophile, *Thermus aquaticus*, cross-reacts only weakly, if at all, even with dimeric malate dehydrogenases. The catalytic activities of the malate dehydrogenases vary over a wide range. Potassium chloride, organic solvents such as acetone, and the protein denaturants urea and guanidine hydrochloride activate a number of the malate dehydrogenases under the assay conditions employed. The diversity among the bacterial malate dehydrogenases, manifested not only in molecular size and subunit structure but also in properties such as catalytic activity and the dependence of this activity on electrolytes, organic solvents, and denaturants, indicates significant structural differences between several of these cognate enzyme species.

An enzyme of the tricarboxylic acid cycle, malate dehydrogenase (L-malate–NAD^+ oxidoreductase, EC 1.1.1.37), occurs in most living systems. The enzyme from animal cells has been investigated extensively, but information about it from bacterial sources, and especially from thermophiles, is relatively scanty. The mammalian and other eucaryotic malate dehydrogenases now appear to have molecular weights in the range (60–70) × 10^3 and to be dimeric with a subunit molecular weight of (33–35) × 10^3 (Banaszak & Bradshaw, 1975). Bacterial malate dehydrogenases exhibit some diversity in molecular size, some being similar to their eucaryotic counterparts and others from *Bacilli* and certain other bacteria being approximately twice as large and apparently tetrameric (Murphey et al., 1967a,b; You & Kaplan, 1975). We recently described simple, efficient methods for the isolation of malate dehydrogenases from a number of mesophilic and thermophilic bacteria, preparatory to a detailed study of these enzyme varieties (Wright & Sundaram, 1979). The present investigation of these malate dehydrogenases has yielded information on their molecular size, subunit structure, amino acid composition, structural homology as assessed by the criterion of immunological cross-reaction, and catalytic activity and the effects on it of salt, organic solvents, and protein denaturants.

Materials and Methods

Pig mitochondrial malate dehydrogenase was obtained from Boehringer Corp.; other chemicals were purchased from various commercial sources. *Neurospora crassa* was grown in a salt medium supplemented with sodium acetate as the carbon source, and an extract of the mycelium was prepared (Sundaram & Fincham, 1964) in 10 mM Tris-HCl buffer, pH 8, containing 1 mM $MgCl_2$ and 1 mM 2-mercaptoethanol.

Isolation of Malate Dehydrogenases. Malate dehydrogenase was isolated from the following bacteria by methods already described (Wright & Sundaram, 1979). Extreme thermophiles: *Bacillus caldotenax* and *Thermus aquaticus* YT-1. Moderate thermophiles: a prototrophic *Bacillus*, referred to here as BI, *Bacillus stearothermophilus* N.C.A. 1503 and N.C.A. 1518 Ra2, *Thermomonospora fuscha* N.C.I.B. 11185, and *Thermoactinomyces sacchari* N.C.I.B. 10486. Mesophiles: *Bacillus subtilis* and *Pseudomonas indigofera*. The enzyme preparations appeared to be homogeneous as shown by the observation that they each yielded a single major protein band after electrophoresis in the native state in polyacrylamide gel or in the denatured state in polyacrylamide–sodium dodecyl sulfate gel. An exception was the enzyme from *T. aquaticus*, which in the native state yielded one major and two minor bands of protein, all of which were enzymically active (Wright & Sundaram, 1979). Malate dehydrogenase was purified from *Escherichia coli* by the method of Murphey et al. (1967a).

Determination of Molecular Weights of Native Enzymes. This was carried out by sedimentation equilibrium centrifugation in a Beckman Model E ultracentrifuge using a 12-mm multichannel equilibrium centerpiece in a cell with sapphire windows. The protein samples were centrifuged at 10 °C in an An-J rotor at a speed of ~8770 rpm. Photographs of interference patterns taken at intervals were analyzed to establish the attainment of equilibrium. Prior to the centrifugation, the enzyme samples were exhaustively dialyzed against 100 mM sodium–potassium phosphate buffer, pH 7; the protein concentration was in the range 2–3.5 mg/mL. Molecular weights were calculated by using Nazarian's (1968) equation according to the method described by Dicamelli et al. (1970). This eliminated the need to determine the meniscus concentration.

An approximate estimate of the molecular weights was made by the technique of gel filtration (Andrews, 1965) through a column of Sephadex G-200 calibrated with marker

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proteins. This provided a quick, independent check on the results obtained by the above method.

Determination of Subunit Molecular Weight. The malate dehydrogenases were denatured by heating at 100 °C for 3–5 min in a medium containing 1% (w/v) sodium dodecyl sulfate, 1% (v/v) 2-mercaptoethanol, and 120 μg/mL protease inhibitor phenylmethanesulfonyl fluoride (Laemmli, 1970). The denatured proteins were electrophoresed in polyacrylamide gels, prepared from 10% (w/v) acrylamide, containing 0.2% sodium dodecyl sulfate (Fairbanks et al., 1971). The molecular weights of the subunits of the malate dehydrogenases were determined from their electrophoretic mobilities related to the mobilities of marker polypeptides (Weber et al., 1972).

Determination of Amino Acid Composition. Malate dehydrogenase samples, after exhaustive dialysis against deionized distilled water and lyophilization, were hydrolyzed by being heated with 5.7 N HCl at 110 °C for 24 h in evacuated, sealed tubes. The hydrolysates were evaporated to dryness in vacuo and the excess acid was removed by repeated addition of water and drying. The hydrolysates were dissolved in citrate buffer, pH 2.2, and amino acid analyses were carried out with a JEOL 6AH automatic analyzer.

Immunochemical Experiments. Enzyme samples each containing ~1 mg of protein were emulsified with Freund's adjuvant and injected intramuscularly into the hindquarters of adult male half-lop rabbits at fortnightly intervals for ~6 weeks. Freund's complete adjuvant was used to prepare the sample for the first injection, but the samples for the subsequent injections were prepared in incomplete adjuvant. Sera were prepared from blood samples drawn from the vein at the edge of the ear, diluted threefold with deionized water, and mixed with DEAE-cellulose equilibrated in 10 mM sodium–potassium phosphate buffer, pH 8 (5 g wet weight of DEAE-cellulose per mL of undiluted serum). The DEAE-cellulose was filtered off and washed with buffer. The filtrate and the washings, which contained the immunoglobulin G fraction, were combined and treated with ammonium sulfate to 65% saturation. The precipitated protein was dissolved in phosphate buffer (pH 7), and the solution was dialyzed against the same buffer. The concentration of immunoglobulin in these preparations was determined from their absorbance at 280 nm. The background malate dehydrogenase activity was largely removed by heating the preparations at 60 °C for 30 min. Any residual activity remaining was precisely determined, and an appropriate correction was applied in the enzyme neutralization tests. A control immunoglobulin fraction was prepared from blood drawn from each rabbit before the start of the malate dehydrogenase injections. This control preparation did not inhibit any of the bacterial malate dehydrogenases investigated here.

Immunotitration experiments were performed as follows. Each malate dehydrogenase preparation was diluted in 100 mM phosphate buffer, pH 7, to an activity level of ~1 unit/mL. A mixture (0.9 mL) consisting of 0.1 mL of the diluted dehydrogenase, 0.3 μmol of NADH, an appropriate amount of immunoglobulin, and buffer (phosphate, pH 7) was incubated for 15 min at 30 °C, and then the malate dehydrogenase activity was determined after the addition of 0.1 mL of 33 mM oxaloacetate.

Protein and malate dehydrogenase assays were carried out as described previously (Wright & Sundaram, 1979).

Results

Native Molecular Weights of Malate Dehydrogenases. The molecular weights of the native malate dehydrogenases derived from the data of the sedimentation equilibrium experiments are presented in Table I. Each of the enzymes falls into one of two categories: one with a molecular weight of ~140 000 and the other roughly half this size. A value of 0.74 mL/g for the partial specific volume, which is generally applicable to globular proteins, was assumed in these calculations. The partial specific volumes for the various malate dehydrogenases calculated from their amino acid compositions (see below) were in the range 0.729–0.751 mL/g. A value of 0.74 mL/g rather than the values actually calculated was used because, for reasons given below, a few of the experimentally determined amino acid contents were possibly slight underestimates and there were no values available for the tryptophan and cysteine contents. Representative plots of log ΔQJ against r^2 from the centrifugation data are presented in Figure 1. All the plots were linear except those for the enzymes from *B. stearothermophilus* N.C.A. 1503 and *T. aquaticus*, which showed a slight deviation from linearity. This finding, in conjunction with the polyacrylamide gel electrophoretic profiles of the

Table I: Molecular Weights of Native Malate Dehydrogenases and of Their Subunits[a]

enzyme source	mol wt of native enzyme: sedimentation equilibrium method	mol wt of native enzyme: gel filtration method	subunit mol wt
BI	140855 ± 637	135000	34000
B. stearothermophilus N.C.A. 1503	137068 ± 925	138000	35000
B. stearothermophilus N.C.A. 1518 Ra2		140000	35000
B. subtilis	138590 ± 953	142000	35000
B. caldotenax	134946 ± 420	124000	35300
T. fuscha	79115 ± 1954	70000	35000
T. sacchari	133609 ± 456	125000	32000
T. aquaticus	85076 ± 1541	71000	36000
P. indigofera		67000	34000

[a] For the calculation of the molecular weights in the sedimentation equilibrium centrifugation method the density of the phosphate buffer–protein solution was 1.0138 g/cm^3 and the partial specific volume was taken to be 0.74 mL/g as explained in the text; molecular weights were derived from the plots of log ΔQJ against r^2 (see Figure 1) by the method referred to in the text. Each value for subunit molecular weight is the average of several determinations; the variation between the replicate values for any malate dehydrogenase species was no greater than 500. Marker proteins used were bovine serum albumin, glutamate dehydrogenase, lactate dehydrogenase, malate dehydrogenase (pig heart), trypsin, and cytochrome *c*.

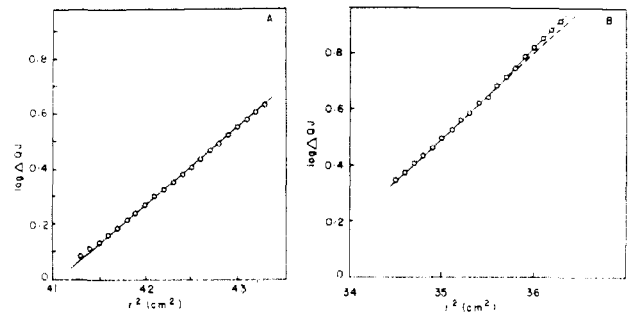

FIGURE 1: Sedimentation equilibrium centrifugation of malate dehydrogenases. The enzymes were contained in 100 mM sodium–potassium phosphate buffer, pH 7. ΔQJ is the fringe rise across a constant interval Q and r is the distance from the center of rotation. The plots are the best-fit lines determined by the method of least squares. (A) BI malate dehydrogenase. Protein concentration was 2 mg/mL and rotor speed was 8769 rpm. (B) *B. stearothermophilus* N.C.A. 1503 malate dehydrogenase. Protein concentration was 3.5 mg/mL and rotor speed was 8776 rpm. The slight deviation from linearity, shown by the broken line, indicates a low level of polydispersity.

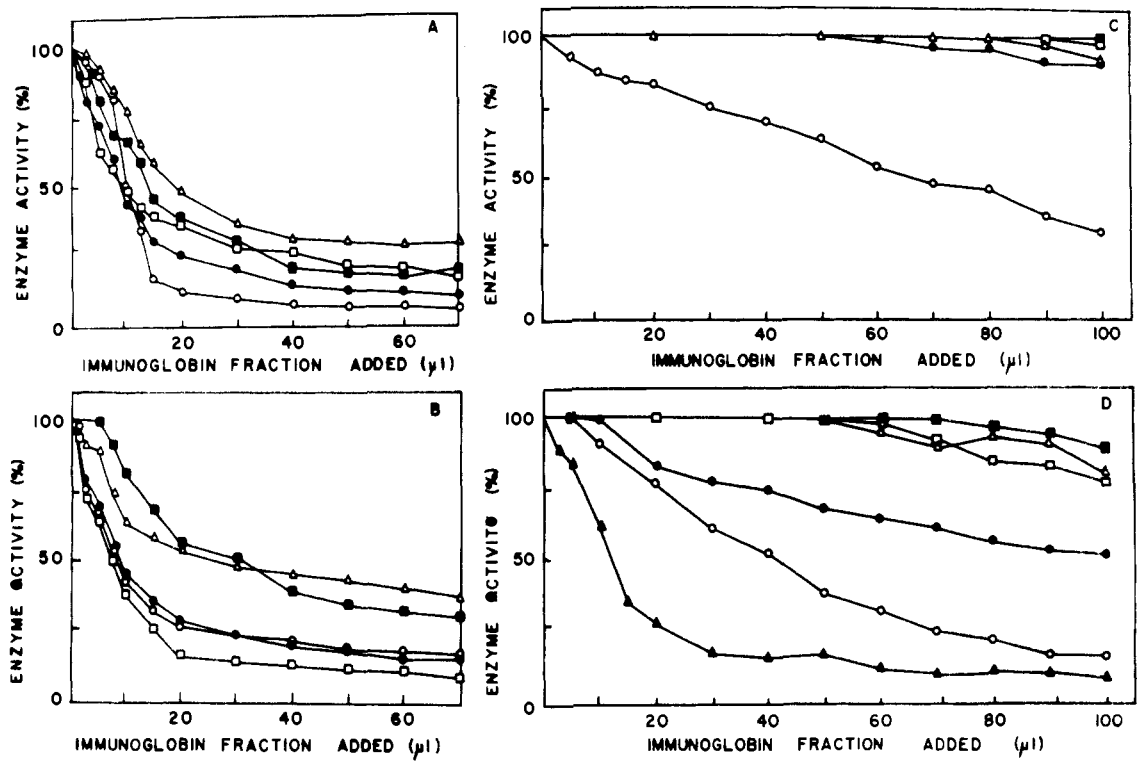

FIGURE 2: Immunotitration of malate dehydrogenases. Purified enzymes were each incubated at 30 °C for 15 min with the amount of immunoglobulin indicated, and the remaining enzyme activity was measured after the addition of oxaloacetate. (A) Immunoglobulin used (23 mg/mL) was that prepared from the antiserum to BI malate dehydrogenase. The enzymes titrated are (○) BI, (△) *B. stearothermophilus* N.C.A. 1518 Ra2, (□) *B. subtilis*, (●) *B. caldotenax*, and (■) *T. sacchari*. (B) The immunoglobulin used (10 mg/mL) was that prepared from the antiserum to *B. subtilis* malate dehydrogenase. The enzymes titrated are (□) *B. subtilis*, (○) BI, (△) *B. stearothermophilus* N.C.A. 1518 Ra2, (●), *B. caldotenax*, and (■) *T. sacchari*. (C) The immunoglobulin used (22 mg/mL) was that prepared from the antiserum to *T. aquaticus* malate dehydrogenase. The enzymes titrated are (○) *T. aquaticus*, (□) *P. indigofera*, (■) *E. coli*, (●) *T. fuscha*, and (△) pig heart. (D) The immunoglobulin used (16 mg/mL) was that prepared from the antiserum to the pig heart malate dehydrogenase. The enzymes titrated are (▲) pig heart, (△) *P. indigofera*, (●) *E. coli*, (■) *T. fuscha*, (□) *T. aquaticus*, and (○) *N. crassa* (unfractionated mycelial extract was used).

native and denatured enzymes (see above), suggests that the malate dehydrogenases used in this study (with the possible exception of those from *B. stearothermophilus* N.C.A. 1503 and from *T. aquaticus*) were in a high state of purity. A further indication of the high degree of purity of two of the enzymes, those from BI and *B. stearothermophilus* N.C.A. 1518 Ra2, came from an examination of these enzymes for their amino-terminal groups; a single amino acid, serine, was detected in either case (Wright, 1978). An approximate estimate of the molecular weights by the gel filtration method (Table I) is in good agreement with the values deduced from the centrifugation experiments.

Subunit Structures. The molecular weights of the subunits produced upon denaturation of the malate dehydrogenases in hot sodium dodecyl sulfate–mercaptoethanol were derived from their electrophoretic mobilities in polyacrylamide–sodium dodecyl sulfate gels and are presented in Table I. In every case only a single major polypeptide band was seen in the gel. This shows that either each malate dehydrogenase contains only one kind of subunit or if it contains nonidentical subunits their molecular sizes are closely similar. From the molecular weights of the native enzymes and of their subunits, the conclusion emerges that the malate dehydrogenases from the *Bacilli* and *T. sacchari* are tetramers, whereas those from *P. indigofera*, *T. fuscha*, and *T. aquaticus* are dimers. The subunit molecular weight in every case falls in the range 32 000–36 000.

Amino Acid Composition. In the present study the contents of half-cystine and of tryptophan were not determined. However, their reported contents in other malate dehydrogenases are very low (Wright, 1978) and therefore the omission of these values in the calculation of the relative proportions of the other amino acids will not have any significant effect. Ideally the values for isoleucine and valine are obtained after hydrolysis of the protein for 72 h and the values for serine and threonine are deduced by extrapolation to zero time of hydrolysis. Since all the data in our study were obtained from 24-h hydrolysates, the contents of these four amino acids determined may be slight underestimates. The results obtained in this study, in conjunction with those reported by other workers on certain bacterial malate dehydrogenases (Murphey et al., 1967a; Biffen & Williams, 1976), suggest that, despite some individual variations, the malate dehydrogenases examined have similar amino acid compositions and that the steadily increasing resistance to thermal denaturation seen in malate dehydrogenase concomitant with a progression from mesophiles through moderate thermophiles to extreme thermophiles cannot be traced to a consistent, significant gradation in the proportion of any amino acid.

Immunochemical Homology. The results of experiments in which the inactivation of a number of malate dehydrogenases by homologous and heterologous immunoglobulins was investigated are summarized in Figure 2. The immunoglobulin from the antiserum to the BI malate dehydrogenase inactivated the homologous enzyme more than 90% and the other tetrameric malate dehydrogenases nearly as efficiently, 72–90% (Figure 2A). It is noteworthy that the *T. sacchari* enzyme, although not produced by a *Bacillus*, was almost as sensitive as any *Bacillus* malate dehydrogenase. None of the dimeric enzymes, namely, those from *P. indi-*

Table II: Catalytic Activities of Malate Dehydrogenases[a]

enzyme source	catalytic act. (10^3) per mole of subunit	catalytic act. (10^3) per mole of native enzyme
Bl	10.0	39.8
B. stearothermophilus N.C.A. 1503	10.0	40.1
B. stearothermophilus N.C.A. 1518 Ra2	10.3	41.0
B. subtilis	12.8	51.3
B. caldotenax	0.4	1.7
T. fuscha	0.3	0.6
T. sacchari	0.8	3.0
T. aquaticus	1.6	3.2
P. indigofera	91.3	182.7
E. coli	19.0	38.0

[a] Catalytic activity is expressed as moles of oxaloacetate reduced per minute per mole of subunit or of native enzyme. The activity of the *E. coli* enzyme is calculated from the data of Murphey et al. (1967a); it was determined at ~25 °C. The activities of the other enzymes are derived from the present study and were determined at 30 °C; they are calculated on the basis of the subunit molecular weights presented in Table I and the number of subunits in the molecule of each enzyme.

Table III: Effect of KCl and of Acetone on the Activity of Malate Dehydrogenases[a]

enzyme source	KCl, enzyme act. (% of control)	acetone: optimal acetone concn (% v/v)	acetone: enzyme act. (% of control)
Bl	1200	5	141
B. stearothermophilus N.C.A. 1503	180		
B. stearothermophilus N.C.A. 1518 Ra2	360	20	160
B. subtilis	540	3	108
B. caldotenax	600	13	316
T. fuscha	2400	20	412
T. sacchari	640	20	397
T. aquaticus	400	3	122
P. indigofera	150		
E. coli	300	5	101

[a] Enzyme activity measured at 30 °C at the optimal concentration of KCl (see text) or of acetone is expressed as the percent of the control activity (activity measured in the absence of KCl and acetone).

gofera, *T. fuscha*, and *T. aquaticus*, was inactivated significantly. The pattern of inactivation produced by the immunoglobulin from the antiserum to the *B. subtilis* enzyme was closely similar (Figure 2B). The antibody to the *T. aquaticus* malate dehydrogenase efficiently inactivated the homologous enzyme but showed little cross-reaction with all other enzyme species tested, tetrameric and dimeric. The results obtained with the dimers are presented in Figure 2C; the enzymes from pig heart, *E. coli*, *P. indigofera*, and *T. fuscha* were inactivated not at all or less than 10% by a level of immunoglobulin that inhibited the *T. aquaticus* dehydrogenase nearly 70%. Antibody raised against the pig heart mitochondrial malate dehydrogenase neutralized the homologous enzyme and the enzyme from the eucaryotic mold *N. crassa* strongly and the *E. coli* enzyme somewhat less efficiently. There was no cross-reaction with the dimeric enzyme species from *P. indigofera*, *T. fuscha*, and *T. aquaticus* at a level of immunoglobulin (50 μL, 0.8 mg) that inactivated the homologous enzyme over 75%; when the level of immunoglobulin was doubled, the extent of cross-reaction increased slightly but was still weak (Figure 2D). There was no effect on the tetrameric malate dehydrogenases.

Catalytic Activity. The catalaytic activities of various malate dehydrogenase species, calculated from the specific activities of the purified enzymes and their molecular weights, are presented in Table II. The catalytic efficiency varies over a wide range irrespective of whether the comparison is made in terms of the native molecule or the basic subunit. The thermophile enzyme species generally, and in particular those from the extreme thermophiles *B. caldotenax* and *T. aquaticus* and from the moderate thermophiles *T. fuscha* and *T. sacchari*, are less efficient than the mesophile species. However, there does not appear to be a strict inverse relationship between the catalytic activity of an enzyme species and the optimum growth temperature of its source organism. Thus, the enzyme from *T. aquaticus*, an extreme thermophile, is appreciably more active than the enzyme from *T. fuscha*, a moderate thermophile. By far the most active species is the dimer from *P. indigofera*. There is no indication, however, that a dimeric species is inevitably more efficient catalytically than a tetrameric counterpart; for instance, the dimer from the moderate thermophile *T. fuscha* is less active than the tetramer from *T. sacchari*, also a moderate thermophile.

Effect of Salt on Catalytic Activity. As shown in Table III, virtually every malate dehydrogenase species examined was activated by KCl. The extent of the activation varied widely, from less than twofold with the enzymes from *P. indigofera* and *B. stearothermophilus* N.C.A. 1503 to over twentyfold with the *T. fuscha* enzyme. The concentration of KCl that produced maximal activation also varied, from 0.15 M for the *P. indigofera* and *E. coli* enzymes to 1.1 M for the *T. fuscha* enzyme; with the other species maximal activation occurred at 0.4–0.8 M KCl. These results do not suggest an absolute correlation between salt activation and thermostability of the malate dehydrogenases, although the most strongly activated enzymes are from thermophiles.

Effect of Organic Solvents on Catalytic Activity. A number of organic solvents including methanol, ethanol, propanol, and acetone were observed to have varying effects on the activities of the malate dehydrogenases. The results obtained with acetone, which activated some of the dehydrogenases more strongly than the other solvents, are presented in Table III. Three enzyme species, those from *T. fuscha*, *T. sacchari*, and *B. caldotenax*, were relatively strongly activated. Activation of a given enzyme by one solvent does not necessarily imply activation by the other solvents. Thus, the *B. caldotenax* enzyme, which was one of the most strongly activated by acetone, was inhibited by methanol. The *T. aquaticus* enzyme was not strongly activated by any of the solvents.

Effect of Denaturants on Catalytic Activity. A number of the malate dehydrogenase species were significantly activated by urea. The *E. coli* enzyme was activated over twofold by 2 M urea; at higher urea concentrations there was a steady decline in activity and complete inactivation occurred at a concentration of 5 M in 10 min at 30 °C. The *P. indigofera* enzyme was activated ~25% by 1 M urea and completely inactivated by 4 M urea. The *B. subtilis* enzyme steadily lost activity upon addition of urea and was completely inactivated by 5 M urea. Several of the thermophile malate dehydrogenases were more strongly activated (Figure 3). The *B. stearothermophilus* enzyme was activated more than threefold and retained its original activity even after 10 min of incubation at 30 °C with 7 M urea; the behavior of the Bl enzyme was rather similar. The *T. sacchari* enzyme was activated about fivefold, the *T. aquaticus* enzyme over twofold, the *B. caldotenax* enzyme nearly sixfold, and the *T. fuscha*

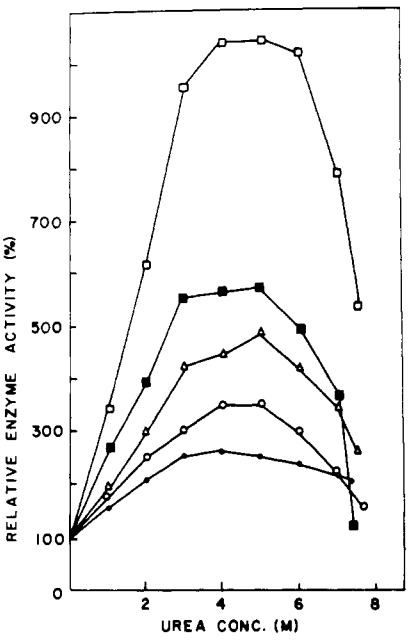

FIGURE 3: Activation of malate dehydrogenases by urea. Purified enzymes were each incubated at 30 °C and pH 7 for 10 min with urea at the concentration indicated, and the enzyme activity was measured. (○) *B. stearothermophilus* 1518 Ra2 enzyme; (■) *B. caldotenax* enzyme; (□) *T. fuscha* enzyme; (△) *T. sacchari* enzyme; (●) *T. aquaticus* enzyme.

enzyme more than tenfold; all of these enzymes retained considerably more than their initial activities even after incubation with 7 M urea for 10 min at 30 °C. The activation was measurably time dependent; maximal activation of the *T. fuscha* enzyme with 3 M urea was achieved after 4 min of incubation.

Guanidine hydrochloride activated the thermophile enzymes, in many instances more strongly than did urea. Maximal activation was observed at a denaturant concentration of 0.5–1 M at 30 °C, and complete inactivation occurred at a concentration of 3–4 M (Figure 4). The BI enzyme behaved similarly to the *B. stearothermophilus* malate dehydrogenase.

Discussion

In this investigation we have examined malate dehydrogenase from a broad spectrum of microorganisms representing the mesophilic, moderately thermophilic, and extremely thermophilic classes and drawn from several genera. A study of a single enzyme from a similar diversity of bacteria does not appear to have been reported before. In an extensive survey of the molecular sizes of malate dehydrogenases from a number of animal, plant, and microbial sources by gel filtration of unfractionated extracts, Murphey et al. (1967b) found that malate dehydrogenases from animal and plant sources and a number of procaryotic and eucaryotic microorganisms had molecular weights of ~62 000 but that *Bacilli* and certain other *Eubacteriales* produced malate dehydrogenases with molecular weights of ~117 000. *B. stearothermophilus* 2184 was the only thermophile included in this survey. Examination of the enzymes purified from *B. subtilis* and *E. coli*, representing the large and small malate dehydrogenases, respectively, in the ultracentrifuge before and after dissociation with acid showed the former enzyme to be a tetramer and the latter a dimer. Our present study reveals that the dimeric and tetrameric species of malate dehydrogenase are both distributed among mesophilic and thermophilic (moderate and extreme) bacteria. Of interest is the finding that the enzyme from *T. sacchari* is a tetramer

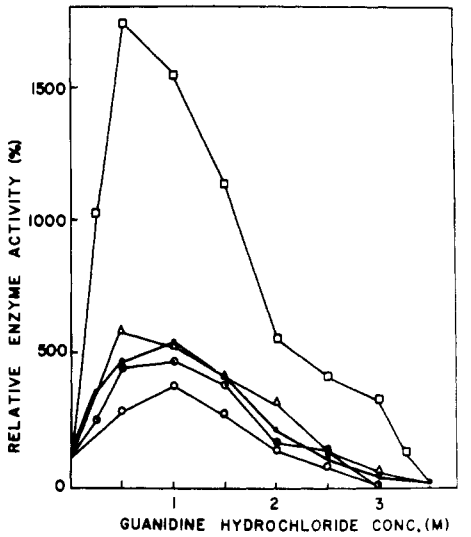

FIGURE 4: Activation of malate dehydrogenases by guanidine hydrochloride. Purified enzymes were each incubated at 30 °C and pH 7 for 10 min with guanidine hydrochloride at the concentration indicated and then assayed for enzyme activity. (○) *B. stearothermophilus* N.C.A. 1518 Ra2 enzyme; (⊙) *B. caldotenax* enzyme; (□) *T. fuscha* enzyme; (△) *T. sacchari* enzyme; (●) *T. aquaticus* enzyme.

whereas that from *T. fuscha* is a dimer, although both of these bacteria are classified as actinomycetes. It has been suggested that malate dehydrogenases are composed of identical subunits of molecular weight 33 000–35 000 (Banaszak & Bradshaw, 1975). The bacterial enzymes studied here, both dimeric and tetrameric, fit in with this pattern (Table I) with the proviso that the identity of the subunits remains to be unequivocally established. Although the native molecular weight of the *T. aquaticus* enzyme, deduced from centrifugation data, is slightly higher than expected (Table I), the subunit molecular weight (Table I) is in the normal range and suggests, as does the result from gel filtration (Table I), that the native enzyme is a dimer with a molecular weight of ~72 000.

The results of the immunochemical experiments suggest a degree of structural specificity in the malate dehydrogenases. Thus, whereas there is free cross-reaction between the tetrameric enzyme species, there is no indication of significant homology between tetramers and dimers, as judged by the criterion of enzyme neutralization. More significantly, the dimeric *T. aquaticus* enzyme shows little cross-reaction even with the bacterial (mesophile and thermophile) dimeric malate dehydrogenases, and the pig heart enzyme, also a dimer, does not cross-react significantly with any of the bacterial dimers with the exception of the *E. coli* enzyme. The cross-reaction with the *E. coli* malate dehydrogenase may be related to the occurrence of this bacterium in the intestinal flora of vertebrates. It must be appreciated that primary structure homology between cognate enzymes, which can be identified by immunochemical cross-reaction between the denatured proteins, may not be detectable in cross-reaction tests between the native proteins (Zakin et al., 1978). Therefore, the extent of primary structural homology between the malate dehydrogenases, especially those that did not cross-react in tests carried out with antisera to the native enzymes, remains to be explored.

Immunochemical homology between thermophile enzymes and their mesophilic counterparts has been studied to a limited extent. Piepersberg et al. (1975) observed significant cross-reaction between the phenylalanine transfer ribonucleic acid ligases from *E. coli* and *B. stearothermophilus*. In the glyceraldehyde-3-phosphate dehydrogenase system, no cross-reaction was seen between the enzymes from *B. stearothermo-*

philus, rabbit muscle, and yeast (Amelunxen & Murdock, 1978). Murphey et al. (1967a) detected cross-reaction between *B. stearothermophilus* malate dehydrogenase and the antiserum to the *B. subtilis* enzyme but not the antiserum to the *E. coli* enzyme. Our study examines cross-reaction within a much wider range of dimeric and tetrameric malate dehydrogenases, mesophilic and thermophilic and produced by different bacterial genera (Figure 2).

The very wide range over which the catalytic efficiency of the malate dehydrogenases varies is noteworthy. Of particular interest is the great disparity in activity between the *T. sacchari* enzyme and the enzymes from the *Bacilli*, BI, and *B. stearothermophilus* (Table II). All of these organisms are moderate thermophiles with similar optimum growth temperatures, and their malate dehydrogenases bear great similarity to one another in molecular size, subunit structure, and immunochemistry. The extremely low activity of the *T. fuscha* enzyme is also of interest. The activation of a number of the malate dehydrogenases by salt and organic solvents is presumably attributable to conformational changes induced by these reagents, and the diversity of response exhibited by the enzymes is indicative of dissimilarities in their conformations. Activation of enzymes by salts is not uncommon and has been observed in other systems including thermophiles (Griffiths & Sundaram, 1973; Fujita et al., 1976). Activation by organic solvents is more unusual but not unknown. The glyceraldehyde-3-phosphate dehydrogenase from the extremely thermophilic *Thermus thermophilus* is activated fivefold by ethanol and to a lesser extent by other alcohols and acetone (Fujita et al., 1976), and the same enzyme from pig muscle is activated about twofold by dimethylformamide (Elodi, 1961). Elodi attributed the activation to a loosening and partial unfolding of the protein structure due to the partial solvation of apolar side chains by dimethylformamide.

The strong activation of several malate dehydrogenases by the protein denaturants urea and guanidine hydrochloride is even more unusual than activation by organic solvents. Here generally the mesophilic enzyme species are less prone to activation than the thermophilic species. Curiously, however, the enzyme from the moderately thermophilic *T. fuscha*, which is the least active catalytically, is the most susceptible to activation by denaturants as well as by salt and organic solvents and the extremely thermophilic *T. aquaticus* enzyme is one of the less strongly activated species.

A salient point to emerge from this study is the diverse nature of the bacterial malate dehydrogenases evident not only in molecular size and subunit structure but also in properties such as catalytic activity and the dependence of this activity on electrolytes, solvents, and denaturants. This suggests that these cognate enzyme species differ significantly in their structural features.

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References

Amelunxen, R. E., & Murdock, A. L. (1978) in *Microbial Life in Extreme Environments* (Kushner, D. J., Ed.) p 250, Academic Press, London.

Andrews, P. (1965) *Biochem. J. 96*, 595.

Banaszak, L. J., & Bradshaw, R. A. (1975) *Enzymes, 3rd Ed. 11*, 369.

Biffen, J. H. F., & Williams, R. A. D. (1976) *Experientia, Suppl. 26*, 157.

Dicamelli, R. F., Holohan, P. D., Basinger, S. F., & Ledowitz, J. (1970) *Anal. Biochem. 36*, 470.

Elodi, P. (1961) *Acta Physiol. Acad. Sci. Hung. 20*, 311.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606.

Fujita, S. C., Oshima, T., & Imahori, K. (1976) *Eur. J. Biochem. 64*, 57.

Griffiths, M. W., & Sundaram, T. K. (1973) *J. Bacteriol. 116*, 1160.

Laemmli, U. K. (1970) *Nature (London) 227*, 680.

Murphey, W. H., Barnaby, C., Lin, F. J., & Kaplan, N. O. (1967a) *J. Biol. Chem. 242*, 1548.

Murphey, W. H., Kitto, G. B., Everse, J., & Kaplan, N. O. (1967b) *Biochemistry 6*, 603.

Nazarian, G. M. (1968) *Anal. Chem. 40*, 1766.

Piepersberg, A., Hennecke, H., Engelhard, M., Nass, G., & Bock, A. (1975) *J. Bacteriol. 124*, 1482.

Sundaram, T. K., & Fincham, J. R. S. (1964) *J. Mol. Biol. 10*, 423.

Weber, K., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26*, 3.

Wright, I. P. (1978) Ph.D. Dissertation, University of Manchester.

Wright, I. P., & Sundaram, T. K. (1979) *Biochem. J. 177*, 441.

You, K., & Kaplan, N. O. (1975) *J. Bacteriol. 123*, 704.

Zakin, M. M., Garel, J., Dautry-Varsat, A., Cohen, G. N., & Boulot, G. (1978) *Biochemistry 17*, 4318.