Thermolability of Malic Dehydrogenase from the Obligate Psychrophile *Vibrio marinus*¹

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ABSTRACT

LANGRIDGE, PATRICIA (Oregon State University, Corvallis), AND RICHARD Y. MORITA. Thermolability of malic dehydrogenase from the obligate psychrophile *Vibrio marinus*. *J. Bacteriol.* 92:418-423. 1966.—The thermolability of malic dehydrogenase in whole cells of *Vibrio marinus* MP-1 grown at 15 C was compared with that of cell-free extracts and partially purified fractions. The intracellular enzyme was found to be stable between 0 C, and the organism's optimal growth temperature, 15 C. In cell-free extracts, considerable lability was noted even at 0 C, and this lability did not increase further until the enzyme was exposed to temperatures above the organism's maximal growth temperature (20 C). Twenty-fold purified enzyme was stable between 15 and 20 C, but both above and below this there was considerable inactivation. A 5-min exposure of both cold- and heat-inactivated enzyme to 15 C allowed reactivation, although to a different extent. Ammonium sulfate was found both to stimulate enzyme activity and to reactivate temperature-inactivated enzyme.

Several reports describing the physiological characteristics and growth of psychrophilic bacteria have appeared in the literature in recent years. Until recently, few of the organisms studied exhibited a maximal growth temperature of 20 C or less, and therefore could not be considered to be "obligate" in their low temperature requirement (3).

The importance of using bacteria which are obligately psychrophilic in nature must be emphasized if the studies are to give any true indication of the physiological nature of psychrophily. Colwell and Morita (2) described the isolation and characterization of a series of marine psychrophiles. These have been designated as strains of *Vibrio marinus*, several of which have maximal growth temperatures of less than 20 C.

Approximately 90% of the marine environment is at a temperature of less than 5 C, and studies of marine microorganisms, which have a growth range allowing proliferation at these temperatures, are essential if we are to make any estimations of the activity of such organisms in

their natural environment. Morita and Albright (5) have shown that *V. marinus* MP-1 grows well at 3 C (that is, at the normal environmental temperature), and produces 10⁹ cells per milliliter in 24 hr. Other studies with this organism have shown that it has an optimal growth temperature of 15 C. It does not grow above 20 C, and incubation above this temperature caused loss of viability.

Further studies (S.M. Robison, M. S. Thesis, Oregon State Univ., Corvallis) were initiated to establish the nature of this heat-induced lethal effect. Oxygen uptake of whole cells at 15 C in the presence of glucose was greatly reduced by treatment at 20.5 C for 80 min compared with the controls. Loss of integrity of the cell membrane was suggested as the cause of the death, as leakage of materials which absorb at 260 mμ occurred at this temperature. As membrane damage could not account for the complete loss of oxygen uptake, the possibility existed that there was a concomitant denaturation of one or more enzymes associated with this uptake.

That the thermolability of a single enzyme is related to loss of cellular function at increased temperatures has been shown by Burton and Morita (1). These studies were performed with a facultative psychrophile, *V. marinus* PS-207, with an optimal growth temperature of 24 C.

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Malic dehydrogenase (MDH) activity decreased to 20% of its original value when cell-free extracts were heated to 30 C for 40 min.

Heat inactivation of formic hydrogenlyase in whole cells of a facultatively psychrophilic strain of *Escherichia coli* was shown to be far greater than in a mesophilic strain under the same conditions (9).

The above studies therefore provided the basis for the present investigations into the thermolability of MDH in partially purified preparations from the obligate psychrophile *V. marinus* MP-1.

MATERIALS AND METHODS

Organism. The marine psychrophile *V. marinus* MP-1 (ATCC 15381), isolated by Morita and Haight (7), was used in these studies. Stock cultures were maintained at 4 C and transferred twice at 15 C prior to mass-culturing at this temperature.

Cell growth. The growth medium consisted of: succinic acid, 1.0 g; NaCl, 15.0 g; Proteose Peptone (Difco), 5.0 g; yeast extract (Difco), 2.0 g; glucose, 2.0 g; Rila marine salts (Rila Products, Teaneck, N.J.), 5.0 g; and FeSO_4 , 0.01 g. The medium was brought to a total of 1 liter with tap water, at pH 7.4. Sterilization was effected by autoclaving at 15 psi for 20 min.

A 36-liter amount of the above medium in a large carboy was inoculated with 3.6 liters of a 12-hr culture of the organism. The culture was incubated with shaking and aeration at 15 C for 21 hr. Harvest was accomplished by centrifugation in a Sorvall RC-2 centrifuge at $20,000 \times g$ and 0 C, with the use of a continuous flow attachment. The cells were washed twice in seawater (26.25 g of Rila salts in 1 liter of distilled water at pH 7.4) and stored at -20 C prior to preparation of cell-free extracts.

For whole-cell studies, the organisms were grown immediately prior to use. A 300-ml amount of the above growth medium was inoculated with 5.0 ml of a 12-hr culture. The cells were incubated for 18 hr at 15 C and aerated by shaking. The cells were harvested as above and suspended in seawater so that a 1:40 dilution gave an optical density (OD) of 0.26 at 525 m μ in a Bausch & Lomb Spectronic-20 colorimeter. The cells were kept on ice between procedures and were used for heat-inactivation studies.

Enzyme preparation. The stored frozen-cell preparation was thawed and 2.6 g (wet weight) of cells was made up to a total of 200 ml in 0.2 M tris(hydroxymethyl)aminomethane (Tris) buffer made with distilled, deionized water and brought to pH 7.4 with sulfuric acid. Purification was carried out as shown in Fig. 1. Cell rupture was carried out in a 10-kc Raytheon sonic oscillator at 0 C. Column chromatography was performed by use of a Pharmacia column (Pharmacia Fine Chemicals, Inc., New Market, N.J.) 1 inch (2.54 cm) in diameter and 18 inches (45.7 cm) in height, packed with Sephadex G-200 gel. The flow was controlled by a Buchler Polystaltic pump and adjusted to give a flow rate of between 1 and 2 ml per hr.

Enzyme assay. The MDH assay described by

FIG. 1. Flow diagram of malic dehydrogenase purification.

Ochoa (8), which measures the oxidation of reduced nicotinamide adenine dinucleotide (NADH_2) when oxaloacetate (OAA) is used as substrate, was used in these studies. The decrease in OD at 340 m μ , observed when NADH_2 is oxidized, was measured by use of a Beckman DU spectrophotometer fitted with a thermospacer connected to a circulating-water bath to give an exact assay temperature of 20 C. The reaction mixtures contained, in 3.0 ml: 1.6 μ moles of OAA, 0.2 μ moles of NADH_2 , and 520 μ moles of Tris adjusted to pH 7.4 with sulfuric acid. Any additions to the assay are stated in the respective figures. Controls omitting NADH_2 , OAA, or enzyme gave no activity. Enzyme units used are as stated by Ochoa (8).

Heat inactivation. A polythermostat constructed by Morita and Haight (7) was used, and the range of temperatures was adjusted between 0 and 40 C depending on the particular requirements of the experiment. The enzyme sample under consideration was pipetted into thermally equilibrated test tubes containing amounts of buffer which would give the required dilution. At given time intervals, samples were withdrawn and assayed directly.

Lysed-cell preparation. Heat-treated whole cells (0.2 ml) were lysed prior to assay by the addition of 1.8 ml of a solution containing 180 mg/ml of Triton

X-100, 360 μ moles of Tris-sulfate (pH 8.6), and 0.8 μ moles of ethylenediaminetetraacetic acid. The mixture was incubated for 5 min at 0 C and assayed.

Protein determination. Protein was estimated by the method of Warburg and Christian (10).

RESULTS

Preliminary investigations were performed to determine the optimal conditions of temperature and pH for the assay of MDH. It was found that at 20 C the reaction continued for at least 2 min in a linear manner, and the highest activity was found at pH 7.4.

Effect of temperature on MDH of whole cells. Washed whole cells suspended in seawater were treated for 1 hr at various temperatures. To find the total quantity of enzyme remaining in the cells, they were treated with Triton X-100 and assayed for enzyme content. There was no change in enzyme activity between 0 and 14 C (Fig. 2), but above this temperature it decreased considerably.

Thermolability studies were also performed on the unpurified cell-free extract (supernatant I in Fig. 1). The results (Fig. 3) indicate that considerable inactivation occurs even at 0 C and that a 10-min treatment at 30 C reduces the activity almost completely.

Enzyme purification. Fractionation of MDH with $(\text{NH}_4)_2\text{SO}_4$ was carried out on this supernatant fluid. Table 1 shows the specific activities

FIG. 2. Malic dehydrogenase activity of washed cells of *Vibrio marinus* MP-1 after exposure to various temperatures for 60 min. Activity of cells treated at 14 C for 60 min was arbitrarily set at 100%. Reaction mixtures contained, in 3.0 ml: OAA, 1.6 μ moles; NADH₂, 0.2 μ mole; Tris-sulfate, 520 μ moles; cellular protein, 0.4 mg. Cells were lysed with Triton X-100 prior to assay at 20 C.

FIG. 3. Effect of temperature on malic dehydrogenase activity of cell-free extracts. Reaction mixture was as in Fig. 2, except it contained 0.44 mg of protein. The cell-free extract contained a protein concentration of 0.89 mg/ml and was treated for various times and temperatures, as shown on the graph, prior to assay at 20 C.

TABLE 1. Specific and total activities of MDH after $(\text{NH}_4)_2\text{SO}_4$ fractionation

Fraction	Total activity (units)	Specific activity (units/mg of protein)
Supernatant I.....	1,555,400	284
0-40% saturation....	707,200	200
40-60% saturation....	986,000	1,074
60-70% saturation....	86,400	300
70-100% saturation...	29,000	36
0-100%, total.....	1,808,400	

of the fractions obtained. The highest specific activity was observed in the fraction precipitating between 40 and 60% saturation. However, a large spread in precipitation was always observed, and consequently the 0 to 60% fractions were pooled and used in further purification procedures. Further examination of Table 1 shows that there was an increase in the total number of units after fractionation. To find whether this effect was due to the salt itself, the assay was performed in buffer containing various amounts of enzyme-grade $(\text{NH}_4)_2\text{SO}_4$ (Fig. 4). The effects of several different ionic environments on the enzyme activity were investigated, but only $(\text{NH}_4)_2\text{SO}_4$ and Na_2SO_4 gave stimulation. All others tried were inhibitory over the same range of concentrations.

As the enzyme had previously been shown to be extremely heat-labile in cell-free extracts, the optimal conditions for stability were found before further purification procedures could be carried out. The enzyme was incubated at 4 C in the various systems for 24 and 45 hr, each at the same protein concentration (Table 2). System 4 of Table 2 was the only one which gave reasonable stability for the required length of time. Ammonium sulfate was therefore used during column chromatography. The specific activities of the most active fractions varied from 2,200 to 2,800 units per mg of protein. All active fractions from the above procedures were stored at -20 C and finally pooled. They were again fractionated with $(\text{NH}_4)_2\text{SO}_4$, and the MDH precipitated between 60 and 70% saturation with a specific activity of 5,124 units per mg of protein, that is, a 20-fold purification. After desalting by use of Sephadex G-25, this fraction was used for thermolability studies.

Inactivation and reactivation of 20-fold purified MDH. Figures 5 and 6 show the thermolability of MDH. The enzyme is optimally stable under these conditions between 15 and 20 C, which is also the organism's optimal to maximal growth range. Incubation at temperatures outside of this range partially inactivate the enzyme. Attempts were made to reactivate the enzyme by placing

FIG. 4. Effect of ammonium sulfate on the activity of malic dehydrogenase. The enzyme fraction used was the 40 to 60% fraction from Table 1. Reaction mixture was as in Fig. 2 and contained 0.34 mg of enzyme protein. The concentrations of ammonium sulfate in the assay buffer are shown above.

TABLE 2. Stability of MDH at 4 C

Additions to system ^a	Per cent activity remaining		
	0 hr	24 hr	45 hr
None, pH 7.4	100	38.0	13.0
None, pH 8.6	100	7.5	5.0
$(\text{NH}_4)_2\text{SO}_4$ 75 μ moles, pH 7.4	100	94.5	75.5
Ammonium sulfate, 75 μ moles, pH 8.6	100	110.0	99.5

^a All systems contained, in 1.0 ml: 200 μ moles of Tris-sulfate, 0.1 ml of the 0 to 60% enzyme fraction (3.4 mg of protein).

FIG. 5. Effect of temperature on partially purified malic dehydrogenase activity. Reaction mixtures were as in Fig. 2 and contained 0.004 mg of protein. Inactivation performed in 0.2 M Tris-sulfate (pH 7.4) and 0.42 mg of protein per ml.

it at 15 C after inactivation at both 0 and 33 C (Fig. 7). The reactivation is much greater in the former case, giving activity values of 15% above those at zero-time. Enzyme inactivated at 33 C could only be reactivated to 50% of the original value.

No inactivation was found when MDH was heated to 40 C in the presence of $(\text{NH}_4)_2\text{SO}_4$. If 3,000 μ moles/mg of protein of this salt is added to the enzyme inactivated at 33 C, complete reactivation to the zero-time value occurred.

DISCUSSION

During preliminary studies, an apparent increase in the MDH activity occurred when whole cells of *V. marinus* were subjected to

FIG. 6. Replot of Fig. 5 showing the effect of 30-min exposure of partially purified malic dehydrogenase to increasing temperatures.

increasing temperature. This effect was also noted by Burton and Morita (1) working with the facultatively psychrophilic strain (PS 207) of the same bacterium, and has since been shown to be due to leakage of the enzyme from the cells at the elevated temperatures and not to temperature-induced stimulation. Study of the effect of heat on whole-cell enzyme content therefore necessitated lysing of the cells with Triton X-100 prior to assay.

At all temperatures above the optimum for growth, there was a loss of activity when the cells were heat-treated for 1 hr (Fig. 2). However, the level of activity in whole cells was not affected at all by the normal environmental temperature of 0 to 15°C. The abnormal heat lability of MDH may explain the reason for the psychrophilic nature of this bacterium.

The effect of temperature on the MDH activity in cell-free extracts was far more drastic. Heating for 10 min at 30°C produced almost complete inactivation, and at 0°C there was substantial inactivation (Fig. 3). The MDH in cell-free extracts from a thermophile, *Bacillus stearothermophilus*, was shown by Morita and Haight (6) to be inactivated at 78°C. The wide difference in thermal lability of the MDH of the psychrophile compared with that of the thermophile definitely indicated that the enzymes are different.

Tris-sulfate was employed throughout this study instead of Tris-HCl. The MDH was found

FIG. 7. Temperature reactivation of malic dehydrogenase. Reaction mixtures were as in Fig. 2 and contained 0.004 mg of protein. At the time indicated by arrows, the enzyme mixtures were placed at 15°C and assayed at 20°C. The 40°C curve represents enzyme heated in the presence of 1,250 μ moles of ammonium sulfate per mg of protein.

to be more stable in the presence of the sulfate ion, and, therefore, our thermostability studies (other than with whole cells) are biased towards a more stable MDH owing to the presence of the sulfate ion (see Fig. 1). Nevertheless, this enzyme displays an abnormally low thermostability.

Malic dehydrogenase from this psychrophile is protected against temperature-dependent inactivation in the whole cell. This is especially evident in the case of cold inactivation of enzyme, which does not occur in vivo. It would seem from Fig. 5 and 6 that the temperature of optimal growth of the organism is in some way related to the range of optimal stability of the enzyme. It is possible that growth at a particular temperature may influence the production of a particular isozymic form which is more stable at that temperature. However, unpublished data indicate that cells grown at 4°C, the organisms environmental temperature, have an enzyme with a temperature activity and lability pattern identical to the enzyme found in cells grown at 15°C. It would therefore seem that such an enzyme is more likely to affect the growth temperature of the organism by restriction of its maximal limit. Very little difference was noted in activities with the nicotinamide adenine dinucleotide analogues when the assays were performed as described by

Moore and Vilee (4), and no conclusive evidence for the existence of isozymic forms of MDH was shown.

Since there is no indication of the presence of isozymic forms of MDH, it appears likely that incubation of the enzyme outside of its optimal temperature range (15 to 20 C) reduces its activity by partially inactivating all of the enzyme molecules rather than selectively inactivating a portion of the molecular population. However, the latter hypothesis has not been excluded.

These results indicate that MDH found in *V. marinus* MP-1 has a high thermolability and is inactivated at low temperatures in cell-free extracts. The enzyme in growing cells is less sensitive to inactivation by variation in temperature. Addition of ammonium sulfate renders the enzyme more stable to temperature-induced inactivation and allows reactivation of the heated enzyme.

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