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Effect of Temperature on Histidine Ammonia-Lyase from a Psychrophile, *Pseudomonas putida*

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Pseudomonas putida was able to grow at 0 C in a complex medium containing L-histidine and to synthesize histidine ammonia-lyase and urocanase. The activity of the former enzyme was assessed between −10 and 60 C in cells and in cell extracts. Activity was maximal from 20 to 35 C. Below 20 C, activity decreased with temperature but, significantly, the enzyme exhibited 30% of its maximal activity at 1.5 C. The temperature response was similar in both intact cells and cell extracts, which indicated that the cell membrane did not significantly limit the entry of histidine at low temperature. Above and below the maximal temperature range, the reduced activity was not caused by irreversible inactivation, as shown by preincubation experiments. Also, when the temperature was rapidly changed from 60 to 30 C during an assay, the reaction rate increased abruptly to the full 30 C activity without a lag. This demonstrated the rapid reversibility of inactivation. The apparent Michaelis constant increased with temperature. As the substrate concentration was decreased, the enzyme activity became less dependent on temperature. The efficiency of substrate entry and catalysis near 0 C are factors in the ability of this facultative psychrophile to grow in a histidine medium at 0 C.

Although numerous studies have been carried out to explain the remarkable heat stability of enzymes from thermophiles (21), there has been less emphasis on the ability of enzymes from psychrophiles to function at low temperatures. Stokes (23) stated that more studies of enzymes isolated from psychrophiles are necessary to elucidate psychrophile enzymology. He suggested that heat sensitivity of enzymes and their ability to function at low temperatures might be related. We have previously shown that *Pseudomonas putida* can grow on slants in medium containing L-histidine at 0 and 20 C, slightly at 37 C, and not at all at 42 C; in addition, urocanase, a histidine-degrading enzyme, functions well at 0 C (10). In this study, the effect of temperature on histidine ammonia-lyase (EC 4.3.1.3; histidase) was investigated. Histidase (20), a heat-stable enzyme of the histidine-degrading pathway, maintains enzyme activity at low temperatures.

MATERIALS AND METHODS

Organism and cultural conditions. *P. putida* A.3.12 was grown at 25 C aerobically in a medium containing histidine (25) as previously described (11). Cells were stored at 4 C (100 mg/ml, wet weight) in 0.01 M potassium phosphate buffer, pH 7.0. Histidase activity was stable during storage. Cells were disrupted by a 1.5-min treatment of the storage cell suspension (3 ml) with the Biosonic ultrasonic probe (Bronwill Scientific Inc.) with intensity at 70.

Growth experiments at 0 C. The growth of *P. putida* in 50-ml Erlenmeyer flasks, each containing 4 ml of histidine medium (25), was studied by measurement of the optical density at 660 nm. Culture flasks were incubated in an ice-water bath. The cell suspensions were assayed for enzyme activity without sonic treatment.

Enzyme assays. Histidase was assayed by the method of Tabor and Mehler (25), except the reduced glutathione (0.1 ml, 0.1 M) was added only to the assay mixture containing the enzyme and was incubated for at least 10 min at 25 C before initiation of the reaction with histidine. After glutathione addition, the reaction mixture was restored to pH 9.2 by the addition of 1 M NaOH. The changes in absorbance at 277 nm were monitored on a recorder. The assays were sometimes carried out in 1 ml (total volume) in water-jacketed microcuvettes, but the concentrations of components were the same as those in the usual 3.0-ml reaction mixture.

It was reported that pyrophosphate inhibited histidase when tris-(hydroxymethyl)aminomethane (Tris) buffer was used for the assay in *Pseudomonas aeruginosa* (13). In the assay of *P. putida*, the activity in pyrophosphate buffer was somewhat higher (28%) than in Tris-chloride buffer (0.05 M). When Tris-chloride and pyrophosphate buffers were both present, the

rates were reduced. Activity with intact cells in Tris-chloride buffer was only 15 to 30% of that with the cell extract. Thus, we used pyrophosphate in the assay because it permitted high activity and gave comparable results with intact cells and extracts. Histidase was easily measured in cell suspensions in pyrophosphate buffer without cell disruption. Histidase activity in intact cells showed a linear dependence on the cell concentration (2 to 20 μliters per 3 ml; data not reported).

The assay of urocanase was described previously (25); we used higher concentrations of substrate (0.067 mM) and phosphate buffer (33 mM) and a recording spectrophotometer. Protein was measured with bovine serum albumin as a standard (14). Crude extract contained about 2 mg of protein per ml.

Rat liver histidase. Frozen rat liver (65 g) was homogenized in a cold Waring blender for 5 min in 260 ml of 0.05 M potassium phosphate buffer, pH 7.4. After centrifugation at 1 C for 30 min at 22,000 × *g*, the supernatant fluid (50 mg of protein per ml) was stored at −20 C.

Temperature. The water-jacketed cuvettes and spectrophotometer sample chamber were held at selected temperatures with a constant-temperature water bath (± 0.01 C control) and circulator. In some experiments, we changed the temperature in a water-jacketed cuvette rapidly by switching to another water bath (see Fig. 4). The temperature was monitored during the assays with thermistor probes (Yellow Springs Instrument Tele-thermometer) submerged in the reaction mixtures. Below 0 C, the coolant was 50% ethylene glycol.

The molar absorptivity (277 nm) of urocanate, the product of the histidase reaction and the basis for the assay, decreased by 7.7% as the temperature was raised from 10 to 50 C at pH 9.2. Thus, the effects of temperature on this enzyme reaction could not be simply attributed to the effect of temperature on the absorbance of the product.

The pH of pyrophosphate buffer, at the concentration of the assay, changed from 9.23 to 9.18 when the temperature was raised from 10 to 50 C. When the bacterial extract and glutathione (adjusted in the reaction mixture to pH 9.2) were added to the buffer, pH decreased from 9.46 to 9.16 as the temperature was raised from 10 to 50 C.

The enzyme preparation in buffer showed positive increments in absorbance at 277 nm compared to buffer alone as the temperature was increased in 5 C steps because of increases in the absorbance of various molecules in the cell extract with temperature. These effects did not contribute error to the assay as these changes were blanked out in the spectrophotometer.

Michaelis constant. The Michaelis constant was measured at different temperatures. The mean of four assays was found for each of eight substrate concentrations. The equation of the best-fit line for a double-reciprocal plot was found by the least-squares method. The Michaelis constant was calculated from the equation. Tris-chloride buffer (0.05 M) was substituted for pyrophosphate buffer because Lessie and Neidhardt had found that *P. aeruginosa* histidase did not follow Michaelis kinetics when pyrophosphate was present (13).

RESULTS

Growth at 0 C. Cells grew in a histidine medium at 0 C in 6 days to maximal turbidity without agitation in a shallow culture (Fig. 1). When yeast extract, glucose with ammonium chloride, or glutamate was substituted for histidine in this medium, cells grew readily in all media in 6 days at 0 C. Under these conditions, cells grew in 1 day at 25 C but failed to grow at 42 C on all four substrates. At 37 C, only slight growth occurred (13 to 14% of the turbidity at 25 C). Therefore these cells are psychrophiles, which have been defined as bacteria that grow well at 0 C within 1 week (23). Because the temperature maximum is about 37 C, the organism is a facultative psychrophile. When histidine was omitted, growth was decreased about 66%. The growth that did occur was attributed to the yeast extract in this medium. Histidase and urocanase, the first two enzymes of histidine catabolism, were formed at 0 C as the bacteria reproduced (Fig. 1). In the maximal stationary phase, the activity of these enzymes decreased even though they were at 0 C.

Effect of assay temperature on histidase activity. Histidase activity in the bacterial extracts had an unusually broad temperature optimum. Between 20 and 40 C, the reaction velocity was relatively independent of the assay temperature (Fig. 2). Rat histidase was studied

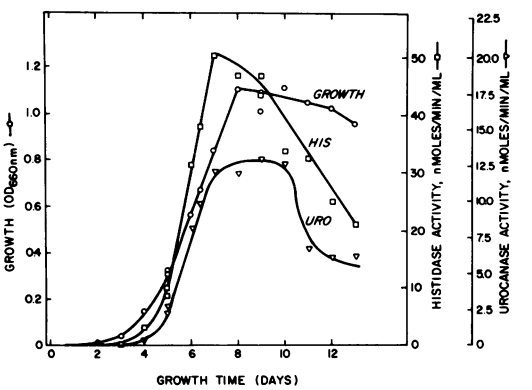

FIG. 1. *Growth of Pseudomonas putida at 0 C on histidine with formation of histidase and urocanase. Histidase and urocanase were assayed with 0.2 and 0.5 ml of culture suspension, respectively. The cells were centrifuged and resuspended in phosphate buffer to remove any substrate or inhibitors remaining in the medium. An optical density (OD) of 1.0 represents 0.467 mg (dry weight) per ml of culture medium.*

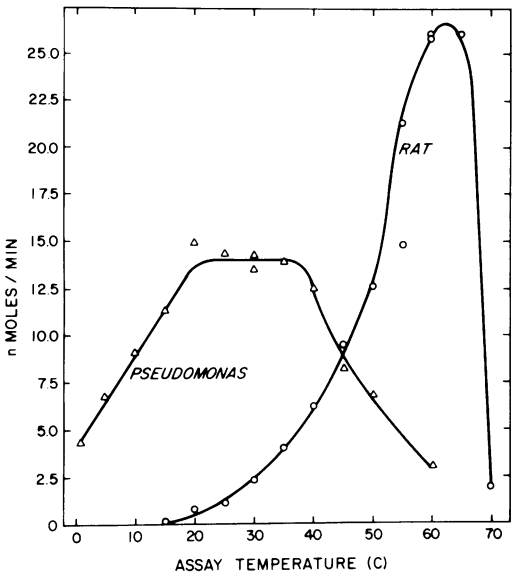

FIG. 2. *Effect of assay temperature on the activity of histidases. Symbols:* Δ, *P. putida extract, 3.3 μliters/ml of reaction mixture;* O, *rat liver homogenate, 20 μliters/ml of reaction mixture.*

for comparison. By contrast, the mammalian histidase activity was strongly dependent on temperature (Fig. 2), with a maximum at 60 to 65 C. The psychrophilic enzyme was a better catalyst at low temperature. Assays below 5 C were conducted with precautions to monitor and avoid condensation on the cuvette. Rat liver histidase had almost no detectable activity at 1.5 C. However, *P. putida* histidase was 30% as active at 1.5 C as at 30 C (Table 1). For experiments below 0 C, part of the water in the assay mixture was replaced by an "antifreeze" (4). Ethanol, methanol, glycerol, ethylene glycol, and propylene glycol inhibited the reaction, but dimethyl sulfoxide did not. An assay mixture (pH 9.2) containing 21% dimethyl sulfoxide remained liquid at −10 C. These experiments showed that the enzyme functioned at −10 C (Table 1). The minimal growth temperature for psychrophiles in general has been placed at −10 C (23). However, we have not attempted to grow *P. putida* below 0 C.

Below the thermal inactivation range, the Arrhenius plot of histidase from rat liver was unmistakably linear from 15 to 55 C. The activation energy was 18.7 kcal/mol. The plots for *P. putida* extracts and intact cells (not shown) were curved in each of 10 experiments, although between 0 and 15 C the plots were almost linear. The problem of interpretation of Arrhenius plots as curved or linear has been discussed (26). Thus, the activation energy for the reaction of *P. putida* histidase was dependent on temperature.

Effect of preincubation of histidases. The possibility that the low activity found at high assay temperatures for *P. putida* histidase (Fig. 2) resulted from irreversible heat denaturation was investigated. The assay mixture, complete except for histidine, was preincubated at various temperatures for 15 min, the duration of a typical assay. The bacterial histidase was very stable to heat (Fig. 3). This histidase lost half of its activity at 82.5 C in 15 min. Histidase from rat liver was less heat stable (Fig. 3). The heat activation of rat histidase (40 to 60 C) was unexpected but reproduced in four experiments. When the bacterial histidase was preincubated at −10 C for 15 min and then restored to 30 C and assayed, there was no irreversible inactivation in the cold (21% dimethyl sulfoxide was used as an "antifreeze" in flasks preincubated at either −10 or 30 C for 15 min and subsequently assayed at 30 C).

Effect of temperature changes during the assay. When the temperature of the reaction mixture was shifted from 30 to 60 C and then returned to 30 C as the bacterial histidase reaction proceeded, the velocity shifted without lag to the rate characteristic of each temperature. A spectrophotometer recorder tracing illustrates the experiment (Fig. 4) for the cell extract. With intact cells (3.3 μliters) in a typical experiment lasting 21 min, a sequence of assay temperatures of 30-60-30-60-30 C resulted

TABLE 1. *Activity of histidase at low temperatures*

Exp	Histidase	Activity[a]		Me_2SO[b]
		Temp (C)	nmol/min	
I	*P. putida* extract[c]	1.5	3.94 ± 0.03	−
		30	13.05 ± 0.13	−
	P. putida cells[c]	2.0	3.31 ± 0.03	−
		30	11.60 ± 0.45	−
	Rat liver[d]	1.5	0.03 ± 0.02	−
		30	3.34 ± 0.16	−
II	*P. putida* extract[c]	0	2.90 ± 0.05	+
		30	14.95 ± 0.60	+
		−10	0.87 ± 0.07	+

[a] Average of three assays ± mean deviation.
[b] Dimethyl sulfoxide (ME_2SO), 21% (vol/vol), present as an antifreeze.
[c] 3.3 μliters in 1.0 ml.
[d] 20 μliters homogenate in 1.0 ml.

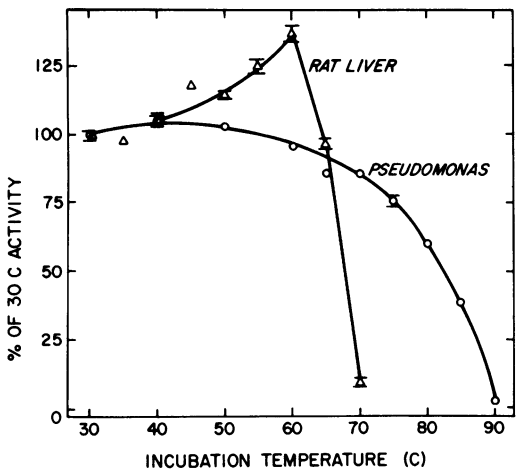

FIG. 3. *Thermal stability of histidases. The histidase was treated 5 min at 25 C with glutathione, preincubated 15 min at different temperatures, cooled rapidly in an ice-water bath, adjusted to 30 C in water-jacketed cuvettes, and assayed. Each point is the average of three assays; mean deviations under 1.25% are not shown by error bars. Symbols: O, P. putida, 3.3 μliters in a total volume of 1.0 ml; Δ, rat liver homogenate, 20 μliters in a total volume of 1.0 ml.*

in rates of 13.7, 3.05, 13.8, 3.02, and 13.4 nmol/min, respectively. The loss of activity of *P. putida* histidase when assayed at temperatures from 40 to 60 C was fully reversible, and the activity changed as rapidly as the temperature. Similarly, when the assay temperature was changed from −10 to 30 C (in the presence of dimethyl sulfoxide) the velocity rapidly increased, and there was no inactivation.

Effect of temperature on Michaelis constant. The apparent K_m for the bacterial histidase was determined at different temperatures. The K_m increased as the assay temperature was raised (Fig. 5).

Effect of substrate concentration on temperature-activity curves. We prepared temperature-activity curves at three lower concentrations of histidine. In the bacterial extracts, the temperature dependence was less as substrate concentration decreased (Fig. 6). Although the histidase was not independent of temperature, at 0.17 mM L-histidine, temperature had little effect. The activity at 2 C was 71% of the activity at 30 C. When a similar experiment was performed with the intact cells (Fig. 6), the response of the histidase in cells to temperature at various substrate concentrations was nearly identical to the response of the extracts.

A small, continuing rate of absorbance change was observed at zero substrate level, as shown in the lowest curve of Fig. 6. At temperatures of 50 and 60 C, the zero substrate rate caused the other curves to level off and even to turn up slightly in the case of 0.17 mM histidine. For 0 to 40 C, the rate of change of absorbance in the absence of histidine does not affect the temperature-activity curves significantly.

The rates for the three lower histidine concentrations (Fig. 6) remained very constant for the duration of assays lasting 15 to 20 min at temperatures of 55 C or below in both cells and extracts. The linear reaction rates indicated

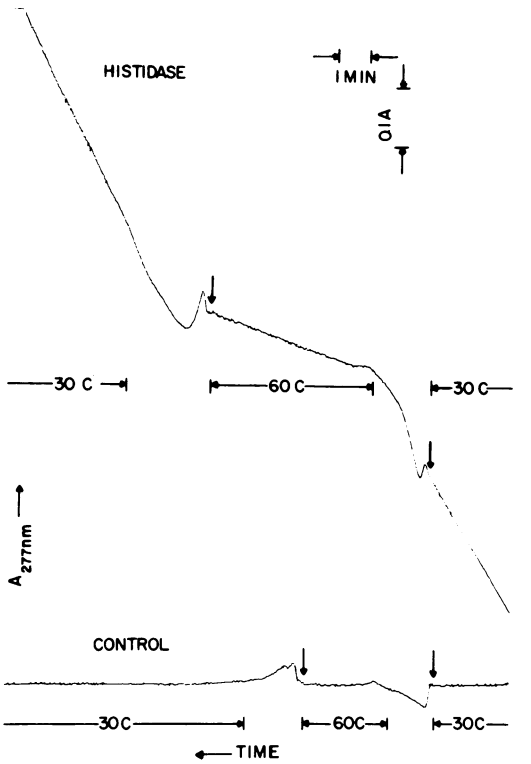

FIG. 4. *Effect of temperature changes during the assay of the bacterial histidase. The figure is a photograph of two recorder charts. Arrows indicate a switch to a water bath of a different temperature (30 or 60 C). The upper (histidase) tracing shows an assay of cell extract, 3.3 μliters/1 ml of reaction mixture. The lower (control) tracing shows the effect of temperature shifts in a water-filled cuvette. Thus, the deflection of the recorder pen seen during temperature transitions reflects instrumental response to the change rather than effects on the reaction. The direction of the deflection depends on the direction of the temperature change.*

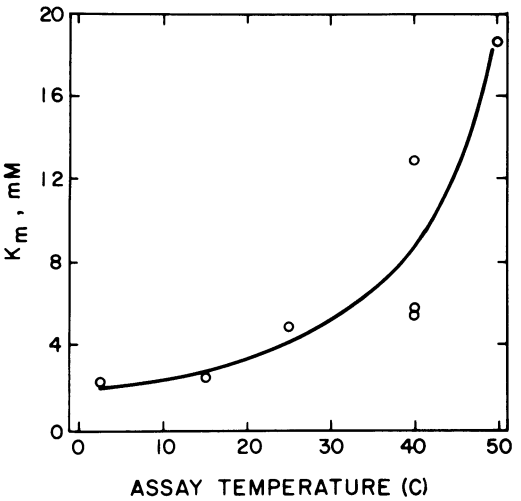

FIG. 5. *Effect of temperature on the Michaelis constant of P. putida histidase. The reaction mixture (3.0 ml) contained 10 μliters of cell extract. Each point represents 32 to 36 histidase assays. The mean correlation coefficient from seven Lineweaver-Burk plots was 0.991 (range, 0.965 to 0.998). Tris-chloride buffer (0.05 M, pH 9.2) was used.*

that product inhibition did not become significant during the assay.

DISCUSSION

The growth experiment (Fig. 1) indicated that *P. putida* grew readily on L-histidine at 0 C and synthesized the enzymes of the histidine-degrading pathway. Histidase activity in *P. putida* had a broad temperature optimum in marked contrast to the rat enzyme (Fig. 2). As the temperature approached either 0 or 60 C, the bacterial enzyme activity was sustained very well (Fig. 6 and Table 1). The temperature-activity curves for *P. putida* cells and extracts were essentially the same (Fig. 6). These results indicate that both the enzyme molecule and the cell membrane are adapted to function in the cold.

Histidase from *P. putida* was very stable to heat (Fig. 3) and cold. Histidase is even more stable when it is not diluted to assay concentration. Tabor and Mehler showed that the histidase was not inactivated at 83 C for 15 min (25) in concentrated solutions, an effect we also noted. The lower activity of the histidase when the assay was performed at 40, 50, or 60 C compared with 30 C (Fig. 2) was not due to irreversible heat inactivation (Fig. 3). Stokes suggested (23) that heat lability and ability to function efficiently in the cold are related properties in psychrophilic enzymes. For this enzyme, *P. putida* histidase, that relationship was not found. However, many psychrophilic enzymes are rapidly inactivated at 30 to 45 C (18, 19, 23, 27, and references given therein). As temperature was changed during the assay of the bacterial histidase, the enzyme activity changed with the temperature without lag (Fig. 4). Whatever the mechanism, the changes do not require substantial time to occur.

Several explanations for nonlinear Arrhenius plots were given by Dixon and Webb (5). Curved Arrhenius plots have been described for several enzymes (1, 6, 12, 15, 17, 24). Svenneby (24) and Paule (17) concluded that, for their proteins, curved Arrhenius plots indicated more than one enzyme form. Brandts interpreted curved plots as evidence that the enzyme is partially denatured at every temperature but least denatured at the temperature of maximal activity (3). We have no evidence based on physical studies of the protein to support this explanation.

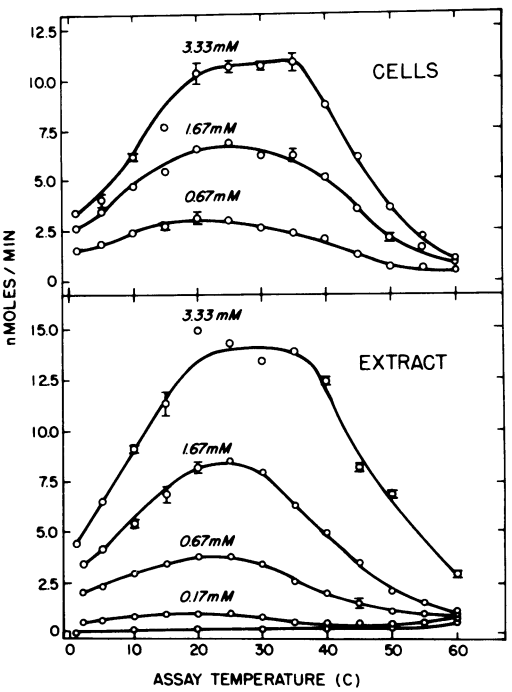

FIG. 6. *Effect of substrate concentration on temperture-activity curves. Each reaction mixture contained 3.3 μliters/1 ml total volume for either intact (above) or sonically treated cells (below); histidine concentration was varied as indicated on each curve. The lowest curve (not marked) represents the data from experiments with no added substrate. Each symbol is the average of three assays; mean deviations under 0.16 nmol/min are not shown by error bars.*

There is an increase of K_m with temperature for various enzymes from poikilothermic animals and bacteria (1, 2, 7-9, 16, 17, 22, 28, 29). Hochachka et al. have discussed the probability that if K_m increases with temperature, the reaction velocity at low substrate concentrations would be relatively independent of temperature (1, 7-9, 22). It was demonstrated that at low substrate concentrations, reaction rates are highly independent of temperature in poikilotherms (1, 7-9, 22, 29). Similarly, the K_m of *P. putida* histidase increased with temperature (Fig. 5), and the temperature dependence of activity was reduced at low substrate concentrations (Fig. 6).

Explanation of the mechanism which permits *P. putida* histidase to adapt to vastly different assay temperatures will require studies on a purified enzyme. The ability of this catalyst and the cell membrane to function at low temperatures are two factors that permit the growth of this microorganism at 0 C in the histidine medium.

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