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CATALYTIC AND THERMODYNAMIC PROPERTIES OF THE UROCANATE HYDRATASE REACTION

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Summary

Urocanate hydratase (4-imidazolone-5-propionate hydro-lyase, EC 4.2.1.49) isolated from *Pseudomonas putida* contains covalently bound α-ketobutyrate as its cofactor. In the process of examining the mechanism by which α-ketobutyrate serves in this capacity, various thermodynamic parameters and temperature effects on urocanate hydratase activity were determined. As the equilibrium constant at 25°C for imidazolone propionate formation from urocanate is approximately 69, regardless of whether urocanic acid or chemically synthesized imidazolone propionate is used as the initial substrate, it is concluded that the reaction is freely reversible. $\Delta G^{\circ\prime}$, $\Delta H^{\circ\prime}$ and $\Delta S^{\circ\prime}$ were −2.5 kcal/mole, +5.2 kcal/mole and +26 cal/deg mole, respectively. Measurement of first-order reaction rates at various temperatures, in order to calculate the Arrhenius activation energy, showed a sharp break in the Arrhenius plot at 29°C. Further examination of this phenomenon by determining $s_{20,w}$ values of urocanate hydratase as a function of temperature revealed a dramatic change at 31°C. Since the enzyme in both experiments reverts to its original state when the temperature is lowered back below the transition point, it is proposed that urocanate hydratase undergoes a reversible conformational change or partial dissociation which affects its catalytic properties in the range of 29—31°C.

Introduction

Urocanate hydratase (4-imidazolone-5-propionate hydro-lyase, EC 4.2.1.49), commonly known as urocanase, has been isolated in pure form from *Pseudomonas putida* and shown to contain covalently bound α-ketobutyrate as a unique prosthetic group [1]. In order to explain fully the mechanism of α-ketobutyrate involvement in the catalytic process, it was deemed necessary to understand more about the reaction catalyzed. Although there is good evidence

that 3-(imidazol-4'-one-5'-yl)-propionate (IPA) is the product of enzymic action on urocanate [2,3], the instability of IPA towards hydrolysis and oxidation has hindered acquisition of knowledge on the details of its formation. This report is concerned with the thermodynamic aspects of the enzymatic hydration and with the effects of temperature on the activity and conformation of the enzyme.

Experimental Methods

Urocanate hydratase preparation and routine assay

Urocanate hydratase was isolated from *P. putida* (ATCC 12633) by the procedure of George and Phillips [1]. The preparations used herein had a specific activity of 1.95 μmoles per min per mg protein, or greater, corresponding to a purity of at least 93%. Spectrophotometric assays were conducted as described earlier [1].

Preparation of 3-(imidazol-4'-one-5'-yl)-propionate (IPA)

The synthetic procedure was based on the method developed by Freter et al. [4] for the synthesis of 4-imidazolone. Formimino-L-glutamate was prepared as described by Tabor and Rabinowitz [5]. Anhydrous β-phenylethyl alcohol (20 ml) was saturated with dry HCl, and 5.75 mmoles of formiminoglutamate added. The resulting solution was then heated to 90°C and held there for one hour. After cooling to room temperature, the solution was diluted to 200 ml with distilled water. Because of the difficulty in isolating the β-phenylethyl diester of formiminoglutamate free of phenylethyl alcohol, cyclization to the imidazolone was performed directly on the mixture. A small sample (1 ml) was used to determine the minimum quantity of 1 M NaOH necessary to produce the maximum increase in absorbance at 260 nm after 1 min. Normally about 60 ml of 1 M NaOH were added per 200 ml of diluted mixture. The alkali was added rapidly and the solution was vigorously shaken for 1 min. At that time, the solution was quickly brought to below pH 2 (13 ml of concentrated HCl), cooled in an ice bath and stored in the dark at 4°C.

For purification of the imidazolone, the solution was applied to a Dowex-50 (H^+) column (2 × 28 cm) equilibrated with 0.1 M HCl at 4°C in the dark. The column was washed with several liters of 0.1 M HCl to remove phenylethyl alcohol until the absorbance at 260 nm reached a minimum. Elution of the product was then achieved with 2 M HCl, and fractions absorbing at 234 nm (usually found between 160 and 425 ml after starting elution with 2 M HCl) were pooled and concentrated by rotary evaporation. This material was the phenylethyl ester of IPA as evidenced by the fact that 234 nm was not its absorption maximum at pH 1 and absorption increased sharply below this wavelength due to the phenylethyl group.

Hydrolysis of the ester was carried out in 1 M HCl for 30 min in a boiling water bath. Rechromatography on Dowex-50 (H^+) (0.8 × 25 cm) as before yielded two compounds having absorption at 234 nm. The first component eluted with 2 M HCl was a small amount of unhydrolyzed ester appearing at 8—16 ml. This material gave a positive ester test [6]. The second component was the desired product and was eluted between 24 and 35 ml; it gave a

negative ester test. This latter material was concentrated by rotary evaporation and dissolved in 0.1 M HCl. Based on the published extinction coefficient of approximately 3000 in acid [3], the yield of IPA was 0.60 mmoles or 10% from formiminoglutamate. Kjeldahl nitrogen analysis, calculating on the basis of a theoretical 17.9% nitrogen, gave the yield as 0.63 mmoles.

The synthetic product had an absorption maximum at 234 nm in 2 M HCl, which shifted to 260 nm when measured at pH 7.2 (0.05 M potassium phosphate) or to 255 nm in 2 M NaOH. NMR spectra were obtained on sample dissolved in 2 M ^{2}HCl in ^2H_2O with a JEOL 100 MHz High Resolution Pulse NMR spectrometer. Signals were seen at 7.68 ppm (singlet), 4.27 (multiplet), 2.30 (multiplet) and 1.97 (multiplet), corresponding respectively to hydrogens at position 2′, position 5′, position 3 and position 2. Values are listed relative to a DSS internal standard.

Equilibrium constant measurements

For the determination of the equilibrium constant for the urocanate hydratase reaction in the forward direction, urocanate (Sigma Chemical Co.) concentrations were estimated spectrophotometrically before and after reaction equilibrium was attained. Urocanate stock solutions were calibrated based on a molar extinction coefficient [7] of 18 800 at 277 nm and pH 7. The reaction product IPA is unstable in air at neutral pH and rapidly forms *N*-formyl-isoglutamine and 4-oxoglutaramate, thus tending to drive reactions to completion [8]. This difficulty was overcome by performing the enzyme reaction under anaerobic conditions. To avoid the necessity of conducting all spectrophotometric measurements (and any required dilutions) in a strict anaerobic environment, samples from the reaction mixture were withdrawn by syringe and rapidly acidified with HCl to pH 1, at which pH IPA is very stable even in the presence of air [3].

The procedure used was as follows. Equal volumes of 1 mM potassium urocanate, pH 7.5, and 50 mM potassium phosphate buffer, pH 7.5, were mixed in a 25 ml serum bottle. Total volume was varied between 1 and 5 ml. The bottle was capped with a rubber septum and then evacated and flushed with argon several times to insure anaerobiosis. An aliquot (usually 25 μl) was withdrawn by syringe and diluted into 25 mM potassium phosphate which had been adjusted to pH 1 with HCl. Readings were made on a Gilford Model 240 spectrophotometer against the appropriate blank. Next, enzyme solution (0.1—0.5 ml in 25 mM potassium phosphate, pH 7.5) containing 0.1 mg of pure urocanate hydratase per ml was added to the anaerobic mixture. Aliquots were again withdrawn, rapidly diluted in acidic solution and read against a blank which contained the same concentration of acidified buffer and enzyme but had no urocanate. Dilutions were such that all readings were within the range of 0.05 to 0.7 absorbance at 277 nm. Constant readings were achieved after 2—6 h, the time varying with the incubation temperature of the reaction mixture. For 25°C, $t_{1/2}$ was estimated to be 10 min. Incubations were performed in a water bath controlled at the stated temperature ±0.05°C. Absorbance measurements were made at 25°C in 0.4 ml quartz microcuvettes of 10 mm path length.

For measurement of the equilibrium constant in the reverse direction, a

similar procedure was followed with these exceptions. A solution of IPA in 0.01 M HCl was standardized at 1.7 mM based on nitrogen content (Kjeldahl method). A portion (0.20 ml) of this solution was placed in a serum bottle containing 0.20 ml of 0.05 M dibasic potassium phosphate. The final pH of this mixture was 7.5. Immediately after mixing, the bottle was capped, evacuated, flushed with argon repeatedly, and placed in the dark in a 25°C water bath. Urocanate hydratase, 2 μl of a 20 mg/ml solution in 25 mM potassium phosphate, pH 7.5, was added to initiate the reaction. Samples (100 μl) were withdrawn at regular intervals, diluted with an equal volume of 2 M HCl and the absorbance read at 277 nm against a blank containing all components except IPA. Incubation was continued for 3 h.

Estimation of the Arrhenius activation energy

Reaction rate measurements were performed in a volume of 1.0 ml. Each assay contained (final concentration): potassium urocanate, 0.10 mM, potassium phosphate, 50 mM, pH 7.5, and urocanate hydratase, 3.4 μg. Absorbance changes were continuously monitored and the initial 2-min rate was taken for calculation purposes. Within that period, all rates were constant for a given temperature, although substrate concentration was less than saturating (K_m = 0.2 mM) [1]. Changes in the reaction pH and extinction coefficient of urocanate with temperature in the range 15 to 40°C were found to have an insignificant influence on the results, hence no corrections have been applied.

Temperature dependence of sedimentation coefficient

A sample of urocanate hydratase was dialyzed extensively against 0.05 M potassium phosphate, pH 7.5, and diluted with the same buffer to give an optical density of 0.7 at 280 nm; this corresponds to an enzyme concentration of 0.6 mg/ml. Ultracentrifugation was performed in a Spinco Model E ultracentrifuge equipped with a photoelectric scanner and multiplexer. Sample and buffer were placed in a 12 mm carbon-filled Epon double sector centerpiece fitted with sapphire windows. Three identical cells were loaded in an An-F-Ti rotor and centrifuged at 60 000 rev./min. Sedimenting boundaries wery monitored at 280 nm at 8 min intervals. Temperatures were measured by the RTIC resistance system and were stable ±0.3°C upon reaching constant speed. For calculation of $s_{20,w}$, density of buffer was measured pycnometrically at each temperature and relative viscosities were determined in a Cannon-Ubbelohde semi-micro viscosimeter immersed in a precision constant temperature water bath maintained at the desired temperature. A partial specific volume of 0.73 ml/g was used for all calculations [1].

Results and Discussion

Examination of the literature reveals a rather wide range of values for the extinction coefficient of IPA at 260 nm in neutral solution [10]. Although our measurements of absorbance were to be made at pH values around 1 and at 277 nm where IPA was reported to have no significant absorption [3], it was necessary to confirm the accuracy of this statement. All previous preparations of IPA have been enzymatic procedures starting from urocanate and have had

TABLE I

MOLAR EXTINCTION COEFFICIENTS* FOR UROCANATE AND 3-(IMIDAZOL-4′-ONE-5′-YL)-PROPIONATE

Wavelength (nm)	Urocanate		IPA	
	pH 1**	pH 7.5***	pH 1**	pH 7.5***
277	16450	18800†	< 2	1160
260	18250	13550	295	1980
234	4180	5620	2860	1850

* $M^{-1} \cdot cm^{-1}$.

** 0.1 M HCl

*** 0.05 M potassium phosphate buffer.

† This value was taken from Tabor and Mehler [7] and was used in the standardization of solutions for other determinations of extinction coefficient.

to distinguish absorption of IPA from that of residual urocanate, enzyme and buffers, as well as to be concerned with stability of IPA under the conditions necessary for its enzymatic formation and spectrophotometric analysis. The chemically prepared IPA appears identical in its ultraviolet absorbance spectrum with enzymatically produced IPA reported by Brown and Kies [3]. Some pertinent estimates of the molar extinction coefficients for urocanate and the chemically prepared IPA are given in Table I. Our values of 2860 and 1980 for the molar extinction coefficients of IPA at its acid and neutral pH absorption maxima, (234 and 260 nm respectively), agree quite well with those reported by Brown and Kies [3], approximately 3000 and 2000, respectively, but differ somewhat from the value of 4000 ± 200 estimated at 264 nm and pH 7.2 by Hassall and Greenberg [11].

Based on the measured extinction coefficients of urocanate and IPA at 277 nm and pH 1, absorbance measurements of a mixture of the two compounds can be used without correction to indicate the concentration of urocanate directly if the ratio of IPA to urocanate is less than 100 : 1.

Estimation of the position of equilibrium for the urocanate hydratase reaction is complicated by the fact that the product of the forward reaction can exist in a tautomeric relationship. Thus the true equilibrium equation is represented as:

$$\text{Urocanate} + H_2O \underset{\text{Urocanate hydratase}}{\rightleftharpoons} \left[\begin{array}{l}\text{3-(imidazol-4′-one-5′-yl)-propionate} \\ \Updownarrow \\ \text{3-(4′-hydroxy-imidazol-5′-yl)-propionate}\end{array}\right]$$

For the determination of an equilibrium constant, the tautomeric forms were treated as a single product, and the equilibrium constant calculated according to the simplified expression:

$$K = \frac{[\text{IPA}]_{eq}}{[\text{Urocanate}]_{eq}\,[H_2O]} = \frac{[\text{Urocanate}]_{initial} - [\text{Urocanate}]_{eq}}{[\text{Urocanate}]_{eq}\,[H_2O]}$$

TABLE II

EQUILIBRIUM CONSTANTS FOR THE UROCANATE HYDRATASE REACTION AT VARIOUS TEMPERATURES

Equilibrium constant is expressed as the molar ratio of IPA/urocanate, irrespective of the direction of measurement. Values are given ± S.D.

Temperature (°C)	Initial substrate	Equilibrium constant
4	Urocanate	35.5 ± 8.8 (3)
15	Urocanate	49.1 ± 8.2 (3)
20	Urocanate	58.2 ± 5.5 (2)
25	Urocanate	67.0 ± 3.3 (16)
25	IPA	72.5 ± 4.4 (4)
27	Urocanate	77.8 ± 5.1 (3)
37	Urocanate	91.2 ± 7.4 (3)

where $[IPA]_{eq}$ actually refers to the sum of the tautomeric forms. In the present determinations, initial and equilibrium concentrations of urocanate were calculated after correcting readings for changes due to initial sample withdrawal and subsequent enzyme addition. If urocanate goes only to the IPA tautomers, a reasonable assumption under anaerobic conditions and with a pure enzyme preparation, then the difference in the amounts of urocanate should be an accurate estimate of product formed. For the purposes of equilibrium constant calculation, water was assigned unit activity.

Values were obtained for the equilibrium constant at temperatures ranging from 4 to 37°C. These results are presented in Table II. For 25°C, an average equilibrium constant of 67 determined in the direction of IPA formation agrees rather closely with the value of 72 calculated for the same reaction from measurements starting with IPA as substrate. This agreement illustrates rather conclusively that the reaction is freely reversible. Moreover, because the calculation based on reverse reaction data requires that the initial IPA concentration be known, agreement of the result with the forward reaction value supports the conclusion that the IPA preparation was relatively pure, at least with respect to other nitrogenous compounds.

Kaeppeli and Retey [12] have provided evidence that the urocanate hydratase reaction product is actually 3-(4′-hydroxy-imidazol-5′-yl)-propionate which is spontaneously converted to racemic 3-(imidazol-4′-one-5′-yl)-propionate. In the light of their conclusion, we must assume that tautomerization of synthetic IPA to the hydroxyimidazolyl form is sufficiently favorable (either spontaneously or enzyme-catalyzed) such that equilibrium of the urocanate hydratase reaction can be achieved without difficulty from IPA.

If an average value of 69 is taken as the equilibrium constant at 25°C, then a $\Delta G^{\circ\prime}$ of −2.5 kcal/mole (−10 600 J/mole) can be calculated for the Gibbs free energy of the forward urocanate hydratase reaction in the standard state at pH 7.5.

Similarly, $\Delta H^{\circ\prime}$, the standard state enthalpy, can be estimated from the van 't Hoff equation for the temperature dependence of the equilibrium constant. A plot of ln K versus the reciprocal of the absolute temperature has a slope of $-\Delta H^{\circ\prime}/R$ if temperature independence of $\Delta H^{\circ\prime}$ is assumed. Data for

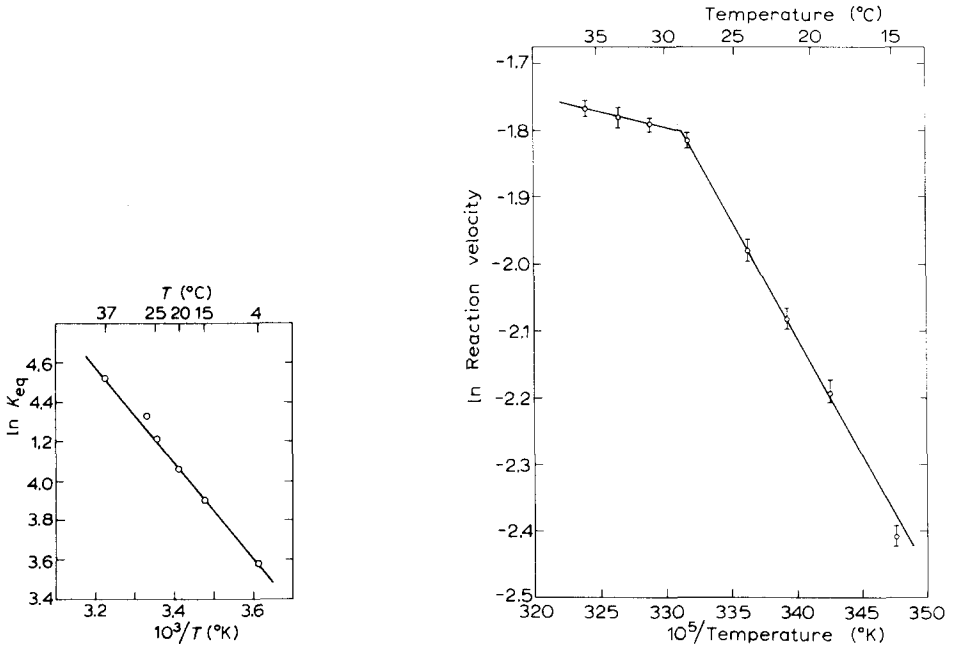

Fig. 1. Determination of ΔH° for the urocanate hydratase reaction.

Fig. 2. Arrhenius plot of observed reaction rates versus (temperature)$^{-1}$. Rates are expressed as absorbance change at 277 nm per min under the specified reaction conditions, ± S.D. for 4 measurements at each temperature.

the urocanate hydratase reaction are plotted in Fig. 1, from which a value of +5.2 kcal/mole (21 800 J/mole) is estimated for $\Delta H^{\circ\prime}$.

The calculation of $\Delta S^{\circ\prime}$ was made from the $\Delta G^{\circ\prime}$ and $\Delta H^{\circ\prime}$ results of −2.5 and +5.2 kcal/mole, respectively. A value of +25 cal/deg mole at 25°C was calculated.

The thermodynamic parameters just estimated suggest that the relatively large positive entropy change is responsible for the overall thermodynamically favorable hydration of urocanate. The large change in $\Delta S^{\circ\prime}$ can be attributed to the formation of the tautomeric equilibrium between the reaction products.

To provide an indication of the unique features of the enzymic process, several catalytic parameters were also investigated. The Arrhenius activation energy, E_a, was determined from observed reaction rates as a function of temperature. First-order reaction rates were measured as described in Experimental Methods with all solutions being equilibrated at the reaction temperature prior to assay and with a thermostatted cell compartment at the same temperature. A plot of ln velocity versus $1/T$ is shown in Fig. 2. There is a distinct discontinuity in the data at 29°C; the activation energy calculated in the region below 29°C is 7.0 kcal/mole. The value above 29°C is 0.9 kcal/mole. All effects noted at higher temperatures (up to 37°C) are completely reversed upon lowering the temperature below 29°C.

Recently Hug and Hunter [13] have published the findings of a similar experiment. They, too, observed that the activation energy is dependent on

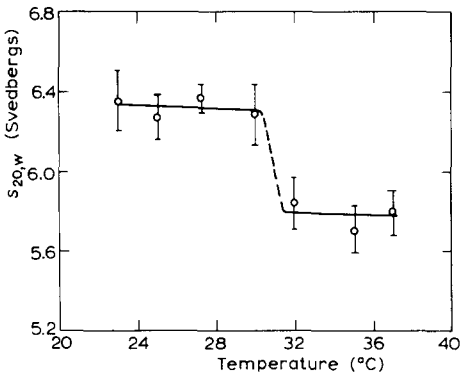

Fig. 3. Effect of temperature on the sedimentation coefficient for urocanate hydratase. Values of sedimentation coefficient are given ± S.D. for 3 observations at each temperature.

temperature but indicated that a smooth curve is obtained, rather than a sharp transition between two linear regions. Regardless of the exact nature of the curves, their result for Arrhenius activation energy at 17.5°C was 8.9 ± 0.6 kcal/mole. This value, as well as our own value of 7.0 kcal/mole, is quite different from that determined for rat liver urocanate hydratase, where an E_a of 27.3 ± 3.9 kcal/mole was calculated [13]. The relatively low Arrhenius activation energy for *P. putida* urocanate hydratase, coupled with the observation that catalytic velocity at 0°C is 30% of that seen at 30°C, led Hug and Hunter [13] to postulate that this behavior contributes to the ability of *P. putida* to grow on histidine as a carbon source at 0°C.

The cause of the sharp change in catalytic properties with temperature was studied further. Sedimentation velocity ultracentrifugation was carried out at a variety of temperatures and the results corrected to 20°C and water as a reference condition. As seen in Fig. 3, sedimentation coefficient ($s_{20,w}$) decreases significantly between 30 and 32°C. The extent of the observed change is only 0.5 Svedbergs, an amount insufficient to be interpreted as a dimer-to-monomer transition, but more likely one due to a limited unfolding of the dimeric structure [9] as temperature increases above 30°C. The conformational change is reversible, since lowering of temperature from 35°C to 25°C restores the higher value (6.3 S) of sedimentation coefficient. While the decrease in sedimentation coefficient observed above 32°C is incompatible with a complete conversion of dimer to monomer, it may reflect a shift in an existing equilibrium between these species. Additional data on weight-average molecular weights as a function of temperature will be required to distinguish between these possibilities.

At the present time it is not possible to conclude whether the small difference in transition point (29°C for catalytic activity, 31°C for conformational change) is significant. Given the uncertainty of absolute temperature calibration of the instruments involved, such a difference is of dubious significance. On the other hand, it is possible that catalytic activity would be more sensitive to minor conformational change than is a gross measurement like sedimentation coefficient. Moreover, because reaction rates were measured at a substrate concentration roughly half-saturating, a significant proportion of the

enzyme exists as the enzyme-substrate complex and thus could result in a slightly different transition temperature. There would seem no question, however, but that the change in catalytic characteristics is closely correlated with an observed conformational change.

Hug and Hunter [13] have previously shown that the K_m for urocanate is not drastically altered over the temperature range of 20 to 40°C. They also concluded that the best explanation for the non-linear Arrhenius plot is a conformational change in the enzyme molecule. Their support for this conclusion is based on difference spectra of enzyme solutions at different temperatures relative to a 10°C enzyme sample. At 30°C and above, a peak in the difference spectrum at 238 nm appeared, attributable to a change in conformation.

Despite the fact that Hug and Hunter [13] obtained a somewhat smooth curve for their Arrhenius plot while our own exhibited a sharp transition temperature, two common interpretations given to such observations are a change in rate-determining step with temperature [14] or a transition between two (or more) stable conformations with differing kinetic properties [15]. While these explanations are closely interrelated, it appears that present data for urocanate hydratase are best understood by the latter interpretation. In the reaction mechanism proposed for urocanate hydratase by George and Phillips [1], a Schiff base is initially formed between α-ketobutyrate and urocanate. Any conformational change which would facilitate the approach of reactive groups on these two molecules can result in a decrease in E_a. Direct evidence to support more efficient active site regions in the enzyme above 30°C is not yet at hand but this may help to explain results obtained by Lynch and Phillips [9] which indicate that one of the two α-ketobutyrate moieties is buried in the tertiary structure of the enzyme and is unreactive to $NaBH_4$ reduction in the native enzyme at 4°C whereas enzyme digested with trypsin can be fully modified by borohydride reduction.

Finally, it should be noted that not only urocanate hydratase but also histidine ammonia-lyase of *P. putida* [16] has been found to exhibit rate transitions as a function of temperature. In the latter enzyme, a sharp break in the Arrhenius plot is observed at 33°C. Thus it appears that both of these enzymes in the histidine utilization pathway may have evolved some common response to temperature changes, but the possible significance of this behavior is presently not understood.

Acknowledgements

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