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SOME CHEMICAL AND PHYSICAL PROPERTIES OF THERMOSTABLE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS*

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SUMMARY

1. Data concerning pH optima, pH inactivation curves, general enzyme kinetics and optimal temperature for assay are presented for crystalline glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:DPN$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12) from *Bacillus stearothermophilus* and from rabbit muscle.

2. Using column density-gradient procedures, the partial specific volume was found to be 0.754 ml/g. The molecular weight for the thermophilic enzyme was calculated as 130 000 by the approach to equilibrium method.

3. Sulfhydryl titration of the thermophilic enzyme revealed 2–3 titratable groups in contrast to 13–14 for the muscle enzyme. *p*-Hydroxymercuribenzoate inhibition of the thermophilic enzyme was completely reversible by cysteine, whereas the muscle enzyme showed only partial reversibility.

4. When dissolved in 8.0 M urea, the thermophilic enzyme showed no change in optical rotation from the native state and under the assay conditions used, there was only a slight loss of enzymic activity; in contrast, the muscle enzyme showed a large negative increase in optical rotation and complete loss of enzymic activity.

5. The thermophilic enzyme is extensively inactivated by DPNH; incubation with DPNH at 60° is necessary to attain the extensive inactivation obtained with the muscle enzyme at 37°.

INTRODUCTION

The crystallization of homogeneous glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:DPN$^+$ oxidoreductase (phosphorylating), EC 1.2.1.12) from *Bacillus stearothermophilus* and data concerning its marked thermostability have been presented in a previous publication[1]. In an attempt to elucidate

Abbreviation: PHMB, *p*-hydroxymercuribenzoate.

a mechanism of thermophily, physicochemical studies have been carried out on this enzyme and such properties compared to the crystalline muscle enzyme. The results of these studies are presented in this paper.

EXPERIMENTAL METHODS AND RESULTS

General methods

Cultural conditions for *B. stearothermophilus*, assay procedures for enzymic activity and protein content, and the purification scheme for obtaining crystalline enzyme have been described[1]. Crystalline rabbit muscle enzyme was prepared by a modified procedure[2]. Greater stability of either enzyme preparation on prolonged storage can be effected by the addition of EDTA to a final concentration of 10^{-3} M.

Effect of pH in the assay system on enzymic activity

In a previous paper[1], the thermophilic enzyme was assayed at pH 8.6 using the method described by KREBS[3] for the crystalline muscle enzyme; under these conditions both crystalline enzymes were almost identical in specific activity. Experiments dealing with the optimal pH conditions for assay of enzymic activity were carried out for both crystalline enzymes using the standard procedure[3] with buffers of different pH values. As seen in Fig. 1, an optimal pH of 8.6 is observed for the muscle enzyme (open circles), as reported previously[4], but the thermophilic enzyme (solid circles) shows greatest activity at pH 10.0.

The buffer employed in the standard assay procedure[3] is pyrophosphate at a concentration of 0.03 M; complete enzymic activation in this assay is achieved in the presence of cysteine. With cysteine activation, the activity of the muscle enzyme is almost identical whether the buffer be pyrophosphate (pH 8.6) at a concentration of

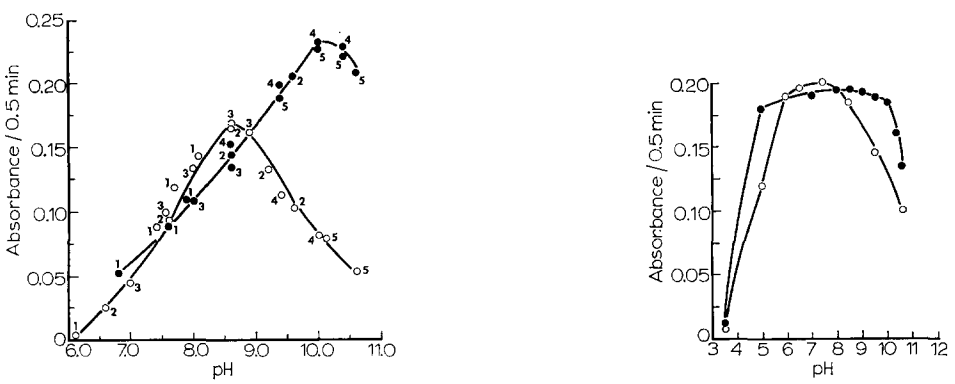


Fig. 1. The effect of pH in the assay system on enzymic activity. The buffers used in the assay system, and their corresponding numbers on the graph are as follows: 1. potassium phosphate, 2. Tris–acetate, 3. pyrophosphate, 4. glycine–NaOH, and 5. carbonate–bicarbonate. ●, thermophilic enzyme; ○, rabbit muscle enzyme.

Fig. 2. The effect of preincubation of enzyme at various pH values on enzymic activity. The buffers used in the preincubation reactions were: glycine–HCl (pH 3.5), sodium acetate (pH 5.0), potassium phosphate (pH 6.0, 6.5, 7.0, 7.5, and 8.0), pyrophosphate (pH 8.6 and 9.0), glycine–NaOH (pH 9.6 and 10.0), and carbonate–bicarbonate (pH 10.4 and 10.6). ●, thermophilic enzyme; ○, rabbit muscle enzyme.

0.03 M or Tris–acetate (pH 8.6) at the same concentration; however, in the absence of cysteine, pyrophosphate is inhibitory when compared to Tris–acetate. Increasing the concentration of any of the buffers to 0.10 M had no effect on the activity of the muscle enzyme with the exception of pyrophosphate which was found to be markedly inhibitory either in the presence or absence of cysteine. The activity of the thermophilic enzyme is nearly identical in either glycine–NaOH (pH 10.0), or carbonate–bicarbonate (pH 10.0), both at concentrations of 0.03 M. Increasing the buffer concentrations to 0.10 M had no effect on the activity of the enzyme with the exception of glycine–NaOH (pH 10.0) in which case enzymic activity was slightly elevated.

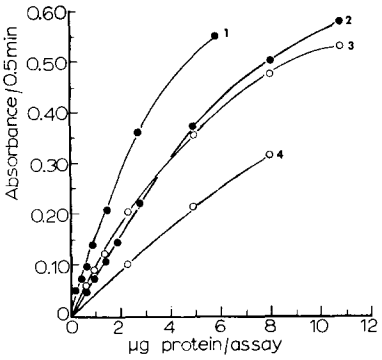
An observation noted on two occasions in this laboratory was significant inhibition of activity of the muscle enzyme (in the presence or absence of cysteine) when assayed using different batches of Tris buffer; such variations have not been noted with any other buffer used. Because of this observation, 0.03 M pyrophosphate (pH 8.6) was selected for assay of the muscle enzyme in all subsequent experiments. In studies with the thermophilic enzyme, 0.10 M glycine–NaOH (pH 10.0) was chosen. Both enzymes freshly prepared exhibit about 70% of their activity when assayed at the optimal pH in the absence of cysteine. Neither enzyme shows demonstrable activity when assayed at the optimal pH if TPN^+ is substituted for DPN^+.

The effect of preincubation of both enzymes with buffers ranging from pH 3.5 to 10.6, followed by assay under the optimal pH conditions for enzymic activity is seen in Fig. 2. Aliquots of each crystalline enzyme were centrifuged at 25 000 $\times$ g for 15 min and the pellets were resuspended in buffers of various pH values all at a concentration of 0.10 M; the resuspended pellets were incubated at 30° for 15 min prior to assay. The thermophilic enzyme (solid circles) shows a wider rangeof stability to pH than does the muscle enzyme (open circles). In neither case does pre incubation at the optimal pH value for enzymic activity result in greatest activity when subsequently assayed under optimal pH conditions.

Effect of enzyme concentration on enzymic activity

The reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase is second order since the rate is a function of substrate and coenzyme. As shown in Fig. 3, the reaction is not linear with increasing concentration of enzyme even with excess substrate and coenzyme as specified in the standard assay system[3]. From curves 2 and 3 in the figure, it is apparent that the thermophilic enzyme (solid circles) and the muscle enzyme (open circles) show similar values over the entire range of enzyme concentration when the buffer in the assay is 0.03 M pyrophosphate (pH 8.6). However, the activity curve of the thermophilic enzyme as shown in curve 1 is markedly higher and has greater linearity when the buffer is 0.10 M glycine–NaOH (pH 10.0), while that of the muscle enzyme under identical conditions shows a marked reduction in activity (curve 4).

Though not shown in the figure, if substrate and coenzyme concentrations are increased to twice the level specified in the standard assay, there is only slight improvement in specific activity and in the linearity of the reaction for the muscle enzyme but the specific activity of the thermophilic enzyme is increased approx. 1.5 times and the range of linearity is similarly increased. Although posing limitations, it was chosen to use the concentrations of substrate and coenzyme as specified in the standard assay[3].



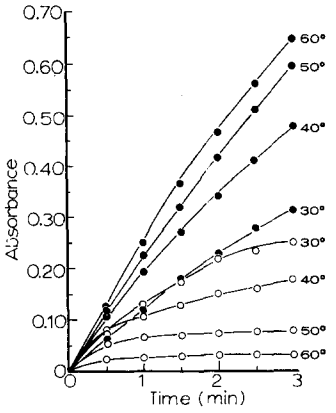


Fig. 3. The effect of enzyme concentration on enzymic activity. The buffer used in curves 1 and 4 was 0.1 M glycine–NaOH (pH 10.0), and in curves 2 and 3, 0.03 M pyrophosphate (pH 8.6). ●, thermophilic enzyme; ○, rabbit muscle enzyme.

Fig. 4. The effect of temperature of the assay system on enzymic activity. ●, thermophilic enzyme; ○, muscle enzyme.

Effect of temperature on enzymic activity

In evaluating the effect of temperature of the assay system on enzymic activity, all assay components were tested for heat stability over the entire range of experimental conditions employed. Temperatures above 60° were not possible because of absorbance changes of assay components in the absence of enzyme. The following modifications in the assay system were made: (1) cysteine was not included because of its instability at elevated temperatures, (2) the DPN^+ and glyceraldehyde-3-phosphate concentration was doubled to achieve greater linearity over the lengthened assay time, and (3) the reaction was initiated with enzyme instead of substrate to prevent inactivation of enzyme during the time needed for assay components to reach the appropriate temperature.

Fig. 4 demonstrates the effect of temperature on the enzymic activity of both crystalline enzymes when assayed under the same conditions. Using pyrophosphate buffer (pH 8.6) in the assay system, both enzymes show almost identical specific activities at 30° as mentioned previously. Under the conditions employed, the reaction rate of the muscle enzyme (open circles) begins to decrease at 40° after 0.5 min and shows marked reduction at 50° and 60°. However, the reaction rate of the thermophilic enzyme (solid circles) continues to increase throughout the range of temperature elevation. Although not shown in the figure, the results obtained with the thermophilic enzyme using glycine–NaOH (pH 10.0) as the buffer in the assay system were essentially the same as with pyrophosphate (pH 8.6) except for increased specific activity at the higher pH.

Since the thermophilic enzyme displays adequate activity at 30° and to avoid non-specific effects of elevated temperatures on assay components, this temperature was chosen for all future experiments.

Partial specific volume

The partial specific volume ($\bar{v}$) of the thermophilic enzyme was determined by

the density-gradient method of OSTER AND YAMAMOTO[5]. This method has been shown by the above authors to be capable of detecting differences as minute as 10^{-7} g/ml. Both NaCl and ^2H_2O–H_2O standards were prepared of known density at 25°. Enzyme at a level of approx. 1.0% by weight of protein was exhaustively dialyzed before use in density-gradient columns. Based on three separate experiments, the $\bar{v}$ was calculated to be 0.754 ml/g.

Molecular weight determination

The Spinco Model E analytical ultracentrifuge was used for the approach to equilibrium method described by ARCHIBALD[6]. Enzyme crystals were dissolved in 0.05 M potassium phosphate (pH 6.5) and then exhaustively dialyzed against the buffer. The protein concentration after dialysis was 0.63%. In the experiments, the analytical D rotor was used at a velocity of 8225 rev./min with the analyzer angle at 80°. Taking an average of several frames during the course of the run, the molecular weight was found to be 130 000 ± 3000.

p-Hydroxymercuribenzoate titration for sulfhydryl groups

The titration of sulfhydryl (–SH) groups for the thermophilic enzyme and the muscle enzyme was carried out according to the spectrophotometric method of BOYER[7] at pH 5.0, and the modified procedure of ALLISON AND KAPLAN[8] at pH 7.0. In acetate buffer (pH 4.6 and 5.0), the thermophilic enzyme shows increasing turbidity with time making –SH titration unreliable. However in phosphate buffer (pH 5.5 or 7.0), from 2–3 titratable –SH groups can be demonstrated based on a molecular weight of 130 000 as reported in this paper. Titration of the muscle enzyme in acetate buffer (pH 4.6 or 5.0) reveals 13–14 –SH groups (based on the currently accepted molecular weight of 138 000 (ref. 9)), which is in agreement with previous reports[10–12]. Only 10– SH groups are titratable in phosphate buffer (pH 7.0) before turbidity obscures the end point of the titration.

Inhibition studies with *p*-hydroxymercuribenzoate (PHMB) were carried out with both enzymes. Reaction mixtures were prepared at 0° and each ml contained 4 mμmoles of enzyme, 5 μmoles of PHMB and 50 μmoles of phosphate buffer (pH 7). Incubations in separate tubes were carried out for 10 min at 0°, 37° and 60° followed by immediate assay for activity under optimal conditions; activity was checked in the presence and absence of cysteine. When assayed in the presence of cysteine, the thermophilic enzyme showed no inactivation at any temperature; in the absence of cysteine the inactivation was 39%, 70% and 80% at 0°, 37° and 60° respectively. Controls without PHMB were unchanged in all cases. On assay of the muscle enzyme in the presence of cysteine, the inactivation was 2% and 57% at 0° and 37° respectively, and 100% inactivation in both cases if assayed without cysteine. Controls without PHMB showed no change at 0° or 37°. Inactivation of the muscle enzyme was complete at 60° with or without PHMB under the conditions employed.

Optical rotatory studies

The optical rotation of the crystalline thermophilic and muscle enzyme was measured in a Rudolph photoelectric spectropolarimeter (Series 200 AS with oscillating polarizer) at the sodium D line and at 5461 Å using the mercury light source. The optical rotatory values and the specific activities of the enzymes in the presence

TABLE I

THE OPTICAL ROTATION AND SPECIFIC ACTIVITY OF CRYSTALLINE GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM *B. stearothermophilus* AND FROM RABBIT MUSCLE

Crystalline enzyme was dissolved in each solvent at a protein concentration of 2.5 mg/ml. The data of Expts. 1 and 2 are representative of the thermophilic enzyme and the rabbit muscle enzyme, respectively.

Expt.	*Solvent*	$-[\alpha]_D^{25}$	$-[\alpha]_{5461}^{25}$	*Specific activity (units/mg)*
1	H_2O	38	50	135
	8.0 M urea	38	50	120
2	H_2O	20	37	85
	8.0 M urea	89	121	0

or absence of 8.0 M urea are presented in Table I. The data indicate that neither enzyme has a particularly large negative rotatory value, but the thermophilic enzyme shows no change in 8.0 M urea while the muscle enzyme shows a large increase in negative rotation. In the case of the thermophilic enzyme, only slight changes in specific activity were noted over a period of several hours in 8.0 M urea whereas the muscle enzyme was inactivated immediately under the same conditions. The optimal conditions for assay of each enzyme as established in this paper were employed. To eliminate the possibility that the thermophilic enzyme may have been inactivated by urea and then reactivated by cysteine, the amino acid was deleted from the assay mixture, but the enzyme still showed only a slight loss in activity.

Effect of DPNH on glyceraldehyde-3-phosphate dehydrogenase from B. stearothermophilus and from rabbit muscle

The extensive inactivation of the rabbit muscle enzyme by DPNH has been

TABLE II

EFFECT OF INCUBATION OF DPNH WITH GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM *B. stearothermophilus* AND FROM RABBIT MUSCLE

Each tube contained in 1.0 ml: approx. 60 mμmoles of enzyme, 50 μmoles of sodium arsenate (pH 8.0) and 2 μmoles of DPNH. Incubation mixtures without DPNH served as controls. Expts. 1 and 2 are representative of the thermophilic and muscle enzymes respectively. Control tubes without DPNH showed little or no change in activity over the entire incubation period, except in Expt. 2 where the control tube at 60° showed a 21% loss at 0.1 h; at zero time, tubes with or without DPNH gave identical values for activity.

Incubation temp.	*Time (h)*	*Inactivation (%)*	
		Expt. 1	*Expt. 2*
37°	0.5	8	22
	1.0	22	50
	2.0	53	98
60°	0.1	—	100
	0.5	36	—
	1.0	78	—
	2.0	96	—

reported[2,10,13]. Since the inactivation is thought to be involved with the active site of the enzyme as postulated in previous papers[10,14], it became of interest to determine if such inactivation could be induced in the thermophilic enzyme which has physicochemical characteristics somewhat diverse from the muscle enzyme. As indicated in Table II, the thermophilic enzyme is also inactivated by DPNH, but inactivation is significantly less than the muscle enzyme if incubation is carried out at 37°. To obtain extensive inactivation of the thermophilic enzyme, incubation with DPNH must be carried out at 60° (thermophilic temperature); the muscle enzyme at the elevated temperature is completely inactivated within minutes in the presence of DPNH. The inactivation of both enzymes by DPNH is apparently irreversible since reactivation by cysteine cannot be effected.

DISCUSSION

The thermophilic enzyme was found to have a pH optimum of 10.0 in contrast to 8.6 for the muscle enzyme. Because of the large number of factors controlling the effect of pH on enzymic activity, the significance of the elevated pH is difficult to evaluate. Perhaps more knowledge about the active site of the enzyme will clarify this parameter. Preincubation of the thermophilic enzyme at various pH values indicated a range of stability extending from pH 5 to 10, while the muscle enzyme showed a narrower range.

The kinetics of the reaction for both enzymes using the methods described indicate linearity (though imperfect) up to an absorbance of approx. 0.200/0.5 min. It should be emphasized that when experiments are based on a comparison of specific activities, the limits of linearity must be observed. There are undoubtedly differences in the binding of substrate and coenzyme to the thermophilic enzyme compared to the muscle enzyme, as indicated by significant increases in rate and linearity following a 2-fold increase in substrate and coenzyme concentration. It was not possible to obtain Lineweaver–Burk plots that yield completely valid K_m values for either enzyme, even by increasing substrate and coenzyme levels to approach pseudo-first-order kinetics.

As the temperature of the assay mixture is increased, the thermophilic enzyme shows increasing enzymic activity. To preclude reagent problems, temperatures above 60° were not possible. Based on the marked thermostability of the enzyme, rate increases would probably have occurred at temperatures much higher than 60°. The muscle enzyme, by contrast shows instability as low as 40°. The concept of "optimal temperature" is one of questionable significance[15]; consequently, an assay temperature of 30° was selected for the thermophilic enzyme since adequate activity is displayed at this temperature. Valid Arrhenius plots could not be obtained from the temperature data because of the kinetics of the reaction.

The $\bar{v}$ of the thermophilic enzyme was calculated to be 0.754 ml/g. This value falls within the upper range of values reported for many proteins[16], and appears to be somewhat characteristic of glycoproteins. It is interesting to note at this time that preliminary data concerning amino acid content indicate the presence of carbohydrate firmly bound to the enzyme. The molecular weight of the thermophilic enzyme was found to be remarkably similar to that of the rabbit muscle enzyme. The significance of the similarity in terms of a mechanism of thermophily is not clear.

In the case of the thermophilic α-amylase (ref. 17) (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1), the molecular weight was found to be considerably lower than its mesophilic counterpart[18].

The thermophilic enzyme was found to contain 2–3 titratable –SH groups, whereas the muscle enzyme has 13–14. Of interest is the fact that of the total –SH groups titratable with the muscle enzyme, only 3–4 are considered to be critical for activity[19]. Experiments are now in progress to evaluate the status of the thermophilic –SH groups. Should all of them prove to be critical for activity, then the active sites of both enzymes would appear to be similar. The thermophilic enzyme shows marked inhibition by PHMB but the inhibition can be completely reversed by including cysteine in the assay system. This is in contrast to the muscle enzyme where PHMB totally inhibits the enzyme and reversibility by cysteine is only partial. It is possible in the case of the muscle enzyme that ancillary –SH groups in the proximity of the active site may be attacked by PHMB leading to losses in activity which cannot be reversed by cysteine.

The optical rotatory studies indicate striking differences between the thermophilic and muscle enzymes. No changes in optical rotation nor loss in enzymic activity were observed with the thermophilic enzyme following treatment with 8.0 M urea, whereas the muscle enzyme showed a large change in optical rotation and an almost immediate loss in activity. It is concluded that while the thermophilic enzyme does not have a large negative rotation characteristic of unfolded or denatured proteins like that demonstrated for the thermophilic α-amylase (ref. 17), it nevertheless exists in a stable conformation that resists structural change by a solvent that breaks hydrogen bonds. This observation is strengthened by the retention of enzymic activity under such conditions. It would appear that the physical state of the molecule is at least one important aspect of the thermostability of this enzyme.

It has been reported[10,13] that DPNH modifies the muscle enzyme conformation resulting in extensive inactivation, and that the –SH groups of this modified enzyme are susceptible to oxidation by molecular oxygen. It was of interest therefore to evaluate the effect of DPNH on the thermophilic enzyme. Extensive inactivation also occurs with the thermophilic enzyme but at elevated temperature, and the inactivation like the muscle enzyme appears to be irreversible since cysteine does not effect any reactivation. The phenomenon of substrate-induced inactivation lends more evidence to the similarity of the active sites of the thermophilic and muscle enzymes.

Studies now in progress are concerned with the active site of the thermophilic enzyme and the possible relationship of firmly bound carbohydrate to a mechanism of thermophily.

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