Purification and Characterization of Thermolabile Glyceraldehyde-3-phosphate Dehydrogenase from the Facultative Thermophile *Bacillus coagulans* KU†

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ABSTRACT: Essentially all homogeneous enzymes that have been isolated from thermophilic bacteria have exhibited intrinsic thermostability in vitro which has provided the basis for the proposed mechanism of how these enzymes function in vivo. However, in this report, glyceraldehyde-3-phosphate dehydrogenase from the facultative thermophile, *Bacillus coagulans* KU, is shown to be significantly less thermostable than this enzyme from mesophilic sources. This observation suggests that the intracellular environment of *B. coagulans* is essential for the function of the enzyme in vivo. A procedure is described for the crystallization of the glyceraldehyde-3-phosphate dehydrogenase from this organism grown at both 37 and 55 °C. The enzyme has a tetrameric molecular weight of 146 800, subunit molecular weight of 36 000, $s^0_{20,w}$ of 7.72 S, f/f_0 of 1.24, an extinction coefficient at 276 nm of 0.80 $cm^2\,mg^{-1}$, and contains firmly bound NAD^+. In contrast to thermoadaptation studies, the growth temperature had no effect on the amount, specific activity, or physicochemical properties of this enzyme. Heat treatment of the homogeneous enzyme in 0.05 M phosphate buffer (pH 7.1) at the thermophilic growth temperature of 55 °C for 5 min resulted in essentially complete inactivation; however, by increasing the ionic strength to 1.8, complete protection was conferred at this temperature. The rate of inactivation of the enzyme in 8 M urea also indicates that the enzyme is less stable than that from other thermophiles. Although the enzyme was still inactivated in 8 M urea when the ionic strength was increased, the rate of inactivation was markedly decreased. The amino acid composition is clearly similar to that from other sources but there is a significant decrease in the basic residues and an increase in serine and glutamate content. Of the physicochemical properties examined thus far, the increase in the glutamate residues is the only difference that seemingly could be related to the conversion of the enzyme from a thermolabile to a thermostable form by increasing the ionic strength.

How thermophilic microorganisms survive at temperatures which normally cause inactivation and/or denaturation of essential macromolecules has been of great interest and a matter of speculation for many years. No single mechanism can account for thermophilic existence but rather a host of molecular changes appear to contribute to the unusual characteristics of these organisms. Of paramount consideration in a mechanism of survival for thermophilic organisms is the heat stability of the cellular proteins. Currently, investigators have purified and characterized approximately 30 proteins from a variety of thermophiles (for reviews see: Singleton and Amelunxen, 1973; Williams, 1975; Ljungdahl and Sherod, 1976). The elevated heat resistance generally exhibited by the purified proteins from caldoactive (extreme) and obligate thermophilic organisms supports the hypothesis that these macromolecules possess intrinsic thermostability.

Previous studies from this laboratory have been directed toward elucidating a molecular basis for the marked thermostability of the glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from the obligate thermophile *Bacillus stearothermophilus* 1503 (Amelunxen, 1966, 1967; Singleton et al., 1969; Amelunxen and Clark, 1970; Amelunxen et al., 1970; Bridgen et al., 1972). Surprisingly, in contrast to the obligate thermophile, this enzyme in crude extracts of the facultative thermophile *Bacillus coagulans* KU grown at either 37 or 55 °C is unusually thermolabile (Crabb et al., 1975). Thus, it seems evident that the mechanism of survival for this facultative thermophile must be different from that proposed for caldoactive and obligate thermophiles.

In light of the extensive physicochemical characterization of the glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus* in this and other laboratories, and the many other studies of this enzyme especially from mesophilic sources, we have extended our comparative studies to this enzyme from *B. coagulans*. This latter system is unique in that it also affords an opportunity for comparative analyses of an enzyme from the same organism grown at both mesophilic (37 °C) and thermophilic (55 °C) temperatures.

To our knowledge, this paper reports the first crystallization and partial physicochemical characterization of an intracellular enzyme from a true facultative thermophile grown at both mesophilic and thermophilic temperatures. The data thus far accumulated demonstrate physicochemical identity for the glyceraldehyde-3-phosphate dehydrogenase synthesized at either temperature. In addition, the amount of enzyme formed in cells grown at 37 or 55 °C is the same. By contrast, Jung et al. (1974), using a facultative strain of *B. stearothermophilus* (ATCC 7954) in thermoadaptation studies, found that the level of glyceraldehyde-3-phosphate dehydrogenase was high in cells grown at 55 °C, but extremely low in cells grown at 37 °C.

Furthermore, it is shown in this paper that increased ionic strength is necessary to confer thermostability to the homogeneous enzyme, which is in agreement with our previous conclusions about this enzyme in crude extracts of *B. coagulans* (Crabb et al., 1975).

Experimental Procedure

General Methods. Cultural conditions for growth of *B. coagulans* KU at 37 and 55 °C, methods of assay for glycer-

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aldehyde-3-phosphate dehydrogenase, protein determinations, and disc electrophoresis procedures have been described (Crabb et al., 1975). Enzyme units are defined by a modified procedure (Amelunxen, 1966). Ultra Pure urea, guanidine·HCl and NaCl, and enzyme grade $(NH_4)_2SO_4$ were obtained from Schwarz/Mann. Dithiothreitol (DTT)[1] was purchased from Calbiochem.

Sodium Dodecyl Sulfate Gel Electrophoresis. Sodium dodecyl sulfate electrophoresis of the enzyme from *B. coagulans* was carried out essentially as described by Weber and Osborn (1969). Trypsin, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, and albumin were utilized as molecular weight standards; 15–70 μg of protein was applied to each gel in these determinations.

Ultracentrifugal Analyses. Sedimentation velocity and high-speed sedimentation equilibrium studies were performed in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics, Rayleigh interference optics, and the RTIC temperature control system. Enzyme was prepared for the ultracentrifugation studies by exhaustive dialysis against 0.1 M sodium phosphate buffer (pH 7.03), containing 10^{-4} M EDTA, or against 6 M guanidine·HCl containing 0.1 M mercaptoethanol (Kawahara and Tanford, 1966).

The sedimentation velocity experiments were conducted in the AN-D rotor using either a double sector 12-mm Kel-F centerpiece or a single sector aluminum centerpiece at 59 780 rpm and 20 ± 1 °C. The boundary position was determined by means of a Nikon microcomparator and the sedimentation coefficients were corrected to a solvent with the viscosity and density of water at 20 °C according to the procedure of Schachman (1957).

In differential sedimentation velocity experiments, an SB-2 syringe microburet was used to add an equal volume of sample to each side of the double sector cell; the samples were adjusted to essentially the same protein concentration.

Sedimentation equilibrium experiments were conducted in the AN-J rotor with a 12-mm double sector Kel-F centerpiece. The molecular weight was evaluated by the meniscus depletion technique of Yphantis (1964) as described for rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by Harrington and Karr (1965), using a 0.02% protein solution, 16 200 rpm, and a temperature of 10.8 °C. The interference and Schlieren patterns were recorded shortly after reaching speed and at various intervals in order to check for cell leakage and attainment of equilibrium. Fringe displacements were measured on a Nikon microcomparator, using 50× magnification.

Spectral Measurements. Samples were prepared for spectral analysis, extinction coefficient, and A_{280}:A_{260} ratios by exhaustive dialysis against 0.2 M ammonium bicarbonate (pH 7.9) containing 10^{-4} M EDTA. The spectra were measured on a Beckman DB spectrophotometer coupled to a log converter and Leeds and Northrup Model G recorder. Dry weights were obtained by lyophilizing 2.00-mL aliquots (0.6 to 1.0 mg/mL) of the samples in preweighed 5-mL Virtis bulb flasks and drying to constant weight in a vacuum oven at 100 °C.

Amino Acid Analysis. The dried samples obtained from the spectral studies were hydrolyzed with constant boiling HCl at 110 °C for 24, 48, or 72 h according to the procedure of Moore and Stein (1963). Amino acid analyses were performed by the procedure of Spackman et al. (1958) with a Spinco amino acid analyzer equipped with a 20-mm flow cell described by Jones and Weiss (1964). Corrections for the decomposition of threonine, serine, and tyrosine were made by linear extrapolation to zero time of hydrolysis, assuming first-order kinetics for decomposition (Hirs et al., 1958). Cysteine was determined as cysteic acid from hydrolysates of performic acid oxidized enzyme (Hirs, 1956) and tryptophan from *p*-toluenesulfonic acid hydrolyzed samples (Liu and Chang, 1971).

N-Terminal Amino Acid Analysis. Following dansylation of performic acid oxidized enzyme, the N-terminal amino acid residue was identified by thin-layer chromatography on polyamide sheets according to the method described by Gray (1972).

Thermostability of Crystalline Preparations. The crystalline enzyme from *B. coagulans* grown at both 37 and 55 °C was adjusted to a protein concentration of about 3.4 mg/mL in 0.1 M sodium phosphate buffer (pH 7.1) containing 10^{-3} M EDTA–DTT, which stabilizes the enzyme against inactivation. The enzyme solution was dialyzed against this buffer to remove residual $(NH_4)_2SO_4$. The dialyzed protein solution was diluted with deionized water–10^{-3} M EDTA–DTT to 1.7 mg/mL, and a buffer concentration of 0.05 M (insufficient ionic strength to confer thermostability). The total ionic strength was then adjusted to 1.8 by the addition (in solid form) of $(NH_4)_2SO_4$ or NaCl. Following heat treatment for 5 min at temperatures ranging from 37 to 65 °C, the samples were cooled rapidly and assayed for enzymic activity. Controls for each of the samples were kept at 4 °C throughout the experiment.

Effect of Urea and Guanidine·HCl on Enzymic Activity. The kinetics of inactivation of crystalline glyceraldehyde-3-phosphate dehydrogenase from *B. coagulans* in 8 M urea were determined at both 4 and 30 °C. Enzyme crystals were dissolved to a protein concentration of about 1.5 mg/mL in the following solvents: 8 M urea–0.05 M sodium phosphate buffer–10^{-3} M EDTA (pH 7.1); 8 M urea–0.05 M sodium phosphate buffer–10^{-3} M EDTA–10% NaCl (pH 7.1); controls for each of the above contained no urea. Similar procedures were used in evaluating the effect of 5 M guanidine·HCl. Samples withdrawn at various time intervals of incubation were assayed immediately under conditions previously described (Crabb et al., 1975) with two modifications. First, the reaction was initiated with enzyme rather than substrate–acceptor, and second, cysteine was eliminated from the assay system. When activity was no longer detectable in the modified assay, the standard assay system containing cysteine was used to check for any reactivation of the enzyme.

Results

Purification and Crystallization of Glyceraldehyde-3-phosphate Dehydrogenase. The following purification procedure is identical for the enzyme from cells of *B. coagulans* grown at either 55 or 37 °C except in the initial step; cells grown at 37 °C were incubated with DNase for 15 min at 25 °C prior to sonication to disrupt a gelatinous consistency characteristic of growth at the mesophilic temperature. Except where noted, the buffer utilized was 0.2 M sodium phosphate–10^{-3} M EDTA (pH 7.1) and centrifugation was carried out at 50 000*g* for 30 min. Enzyme grade $(NH_4)_2SO_4$ was used throughout, and the percent saturation calculated by the formula of Kunitz (1952). Supernatant fluids were clarified when necessary by filtration through glass wool.

Step 1. Washed packed cells (from a 13-L culture in late exponential phase) were suspended in 80–90 mL of deionized H_2O (at 10^{-3} M EDTA–DTT) and sonicated for 5 min in a Branson sonifier (Model W 185), and the preparation was centrifuged for 60 min.

Step 2. The supernatant fluid was brought to 52% of satu-

[1] Abbreviations used are: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NAD^+, oxidized nicotinamide adenine dinucleotide.

TABLE I: Purification Scheme for the Crystallization of Glyceraldehyde-3-phosphate Dehydrogenase from *Bacillus coagulans* Grown at 37 and 55 °C.

Fraction	Total protein (mg)	Sp act.[a]	Recovery (%)
1. Crude extract	3938	5	
2. S.F.[b] at 52% $(NH_4)_2SO_4$	1814	11	100
3. S.F. at 70% $(NH_4)_2SO_4$	684	29	99
4. R.P.[c] of 90% $(NH_4)_2SO_4$ fraction	361	40	72
5. Bio-Gel A-1.5m	222	55	61
6. R.P. of 90% $(NH_4)_2SO_4$ fraction	198	52	52
7. S.F. of 1.5 vol of sat. $(NH_4)_2SO_4$ fraction	143	73	52
8. R.P. of 2.5 vol of sat. $(NH_4)_2SO_4$ fraction	130	68	44
9. Sephadex G-100	88	105	46
10. R.P. of 90% $(NH_4)_2SO_4$ fraction	85	108	46
11. 1st crystals	55	109	30
12. 2nd crystals	42	109	23

[a] Defined as micromoles of NAD^+ reduced minute^{-1} (mg of protein)$^{-1}$. [b] Supernatant fluid. [c] Resuspended precipitate.

FIGURE 1: Crystalline glyceraldehyde-3-phosphate dehydrogenase from *Bacillus coagulans* at a magnification of 165×.

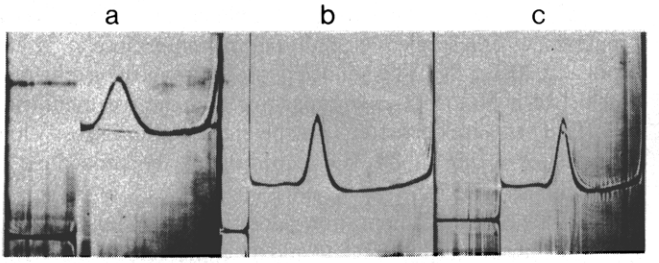

FIGURE 2: Sedimentation velocity patterns of crystalline glyceraldehyde-3-phosphate dehydrogenase from *Bacillus coagulans:* (a) after 5.5 h in 6 M guanidine·HCl and a concentration of 8.5 mg/mL; (b) after 44 min in 0.1 M phosphate buffer (pH 7.03) and a concentration of 6.8 mg/mL; (c) after 40 min in 0.1 M phosphate buffer (pH 7.03) with the same volume and concentration (8.8 mg/mL) in each side of a double sector cell containing enzyme from cells grown at 37 and 55 °C.

ration by the addition of saturated $(NH_4)_2SO_4$ (pH 7.5), allowed to stir for 30 min at 4 °C, and centrifuged.

Step 3. The supernatant fluid was brought to 70% of saturation by the addition of solid $(NH_4)_2SO_4$, allowed to stir 30 min at 4 °C, and centrifuged.

Step 4. The supernatant fluid was brought rapidly to 90% of saturation by the addition of solid $(NH_4)_2SO_4$, and centrifuged.

Step 5. The pellet was resuspended in buffer to about 35 mg of protein/mL and applied to a Bio-Gel A-1.5m (200–400 mesh) column (2.0 × 45 cm) equilibrated with the same buffer, and the effluent was monitored continuously at 280 nm; the flow rate was 20 mL/h, and the temperature was 8 °C.

Step 6. Fractions containing enzyme activity (except the trailing edge) were pooled, brought rapidly to 90% of saturation with solid $(NH_4)_2SO_4$, and centrifuged.

Step 7. The pellet was resuspended in buffer to 20 mg of protein/mL, followed by the dropwise addition of 1.5 vol of saturated $(NH_4)_2SO_4$ (pH 6.6); after incubation for 1 h at 4 °C, the preparation was centrifuged.

Step 8. The 1.5-vol supernatant fluid was brought to 2.5 vol with saturated $(NH_4)_2SO_4$ (pH 6.6); after incubation for 4 h at 4 °C, the preparation was centrifuged.

Step 9. The pellet was resuspended in 2–3 mL of buffer and applied to a Sephadex G-100 column (2 × 90 cm) equilibrated with the same buffer; conditions were as in step 5.

Step 10. All fractions of the first major peak (except the trailing edge) were pooled, rapidly brought to 90% of saturation with solid $(NH_4)_2SO_4$, and centrifuged at 105 500*g* (R_{max}) for 15 min; the presence of a very fine precipitate necessitated the use of higher centrifugal force to pellet the enzyme.

Step 11. The pellet was resuspended to 14 mg of protein/mL in 0.1 M sodium phosphate buffer–10^{-3} M EDTA (pH 6.6) followed by the dropwise addition of saturated $(NH_4)_2SO_4$ (pH 6.6) to the faintest sign of turbidity (about 1.8 vol); rapid crystallization can be effected using 3 cycles of alternate incubation of the preparation at room temperature and 4 °C for 20 min.

Step 12. In subsequent recrystallization of the enzyme, the crystals were centrifuged at 12 000*g* for 30 min, and step 11 was repeated.

A representative purification procedure is summarized in Table I. The percent recovery is based on the total units in step 2 because of a very active NADH oxidase in the crude extract. The specific activity of the crystals is essentially the same from preparation to preparation with cells grown at either temperature, and is comparable to that of the glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus* (Amelunxen, 1975). The pH optimum for activity is 8.6, which is the same as that reported for most other sources of the enzyme. Morphologically, the crystals (Figure 1) are identical from cells grown at 37 and 55 °C, and are similar to those of the enzyme from *B. stearothermophilus* (Amelunxen, 1966); the larger crystals are about 4 × 100 μm.

Homogeneity. Although some tests for homogeneity suggested contamination, the combination of tests indicated that the "contaminants" are generated from the enzyme. Disc gel electrophoresis in 7% acrylamide–Tris·glycine buffer (pH 9.3), using 3 mA/gel, produced two major bands and one minor band, but reelectrophoresis of either major band also gave the same three bands. Using 70 μg of protein/gel, a minor higher molecular weight band could be seen in sodium dodecyl sulfate electrophoresis, whereas sedimentation velocity in guanidine·HCl (Figure 2a) showed only one peak. Sedimentation velocity of the native enzyme (Figure 2b) at higher protein concentrations revealed a small second component that sedimented about one-half the rate of the enzyme. This component

must have been generated from the enzyme since the Sephadex G-100 column would have removed a contaminant of this size, and rechromatography of freshly crystallized enzyme on the Sephadex G-100 column gave a single symmetrical peak. Additional evidence for homogeneity are constancy of specific activity upon recrystallization, a linear relation of log fringe displacement vs. $(r)^2$ from sedimentation equilibrium data, and the presence of a single N-terminal amino acid residue.

Molecular Weight and Sedimentation Studies. Differential sedimentation velocity experiments using a double sector cell containing the glyceraldehyde-3-phosphate dehydrogenase from *B. coagulans* grown at both 37 and 55 °C gave a single boundary in the centrifuge (Figure 2c), indicating that the enzyme formed at either temperature has the same size and shape. A similar experiment in which the enzyme from cells grown at 55 °C and rabbit muscle enzyme were simultaneously centrifuged also showed a single sedimentation rate.

Sedimentation of the native enzyme at several protein concentrations (c) showed a linear dependence of $s_{20,w}$ on protein concentration. Least-squares analysis gave the equation: $s_{20,w} = 7.72\ \mathrm{S} - 0.087(c)$. An $s^0_{20,w}$ of 7.72 S is identical with the previously reported value of 7.71 S for the rabbit muscle enzyme in a similar solvent at neutral pH (Fox and Dandliker, 1956), but somewhat higher than the value of 7.17 S reported for the enzyme from *B. stearothermophilus* (Singleton et al., 1969). Calculation of the frictional ratio, f/f_0, from the molecular weight and $s^0_{20,w}$, gave a value of 1.24 which is identical with the value obtained for the rabbit muscle enzyme based on a molecular weight of 145 000 (Harrington and Karr, 1965), and a diffusion coefficient of 4.97×10^{-7} cm^2 s^{-1} (Fox and Dandliker, 1956).

Sedimentation in 6 M guanidine·HCl gave a single symmetrical boundary with an $s_{20,w}$ of 1.23 S at an enzyme concentration of 8.5 mg/mL (Figure 2a). This is in excellent agreement with the calculated value of 1.21 S for the muscle enzyme at this concentration, which was determined to have a subunit molecular weight of 36 000 (Harrington and Karr, 1965). The subunit molecular weight was confirmed by showing a linear relationship of mobility of known proteins in sodium dodecyl sulfate gel electrophoresis vs. log molecular weight and the identity of migration of the enzyme from *B. coagulans* and rabbit muscle.

In addition to the sedimentation coefficients of the enzyme in the presence and absence of guanidine·HCl, the sedimentation equilibrium studies showed that the native enzyme is a tetramer with a molecular weight of 146 800 ± 3000. The molecular weight was calculated from the slope of a plot of log fringe displacement vs. radial distance squared and the equation:

$$M = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}r^2}$$

where R = gas constant, T = absolute temperature (284 K), ρ = density of solvent (1.0086 g/mL), ω = angular velocity in radians/second at 16 200 rpm, $\bar{v}$ = partial specific volume (0.734 mL/g, based on the amino acid composition), c = protein concentration expressed in fringe displacement, and r = radial distance from the center of rotation. A plot of log fringe displacement (9 to 90 μm) vs. radial distance squared (48.8 to 49.8 cm^2) was linear; $r_b^2 = 49.9$ cm^2.

Extinction Coefficient and NAD$^+$ Content. The spectrum of the glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle and *B. coagulans* (grown at either 37 or 55 °C) exhibits an absorbance maximum around 275–276 nm which yields an extinction coefficient of 1.03 ± 0.04 and 0.80 ± 0.06 cm^2 mg^{-1}, respectively. The value for the rabbit muscle enzyme is consistent with the previously reported E_{276} of 1.06 (Murdock and Koeppe, 1964), whereas the lower value for the enzyme from *B. coagulans* can be attributed to the decreased tryptophan content as shown below for the calculated extinction coefficient at 280 nm. The A_{280}:A_{260} ratio of 1.28 for the enzyme from *B. coagulans* grown at 37 and 55 °C clearly indicates that the enzyme contains firmly bound NAD$^+$. Based on studies with the rabbit muscle enzyme by Murdock and Koeppe (1964), a ratio of 1.28 would represent about 3 equiv of bound NAD$^+$. However, the tryptophan and tyrosine content of the enzyme from *B. coagulans* should increase the A_{280}:A_{260} ratio. In addition, the calculated E_{280} (cm^2 mg^{-1}) for the apoenzyme is only 0.61, whereas the apoenzyme plus 4 equiv of NAD$^+$ is 0.73, which compares favorably with the experimentally determined value of 0.75. These calculations were based on the molar absorbancies at 280 nm of 1150 for tyrosine and 5500 for tryptophan (Wetlaufer, 1962) and the molar absorbancy at 260 nm of 18×10^3 and A_{280}:A_{260} of 0.23 for NAD$^+$ (Siegel et al., 1959). These data and the knowledge that the enzyme isolated from most other sources contains 4 equiv of tightly bound NAD$^+$ are indicative that the enzyme from *B. coagulans* also contains 4 equiv of coenzyme.

Amino Acid Analysis. The results of the amino acid analyses given in Table II show that the composition of the enzyme from *B. coagulans* grown at either 37 or 55 °C is identical. The amino acid residues comprise 91% of the dried samples. The remainder of the sample can be accounted for by presumptive evidence that the enzyme contains 4 equiv of bound NAD$^+$, and that drying the samples from an ammonium bicarbonate solution (pH 7.9) would result in salt formation with lysine, arginine, and the acidic residues. Since only a trace of cystine was detected on acid hydrolysis of the native enzyme, the cysteic acid determination represents cysteine. Dansylation revealed that alanine was the only N-terminal amino acid residue.

Stability of Crystalline Enzyme. Unlike the glyceraldehyde-3-phosphate dehydrogenase from other thermophilic sources, this enzyme from crude extracts of *B. coagulans* was shown to be unusually thermolabile (Crabb et al., 1975). Since other components in the crude extracts could be involved in the inactivation, heat stability studies were conducted on the crystalline enzyme. Another obvious difference is the instability of the crystalline enzyme from *B. coagulans* in the cold in deionized water or in buffers that are less than 0.1 M. As shown in Figure 3, the enzyme from *B. coagulans* grown at either 37 or 55 °C is not stable in dilute buffer at the thermophilic growth temperature of 55 °C since 96–98% of the enzyme activity is destroyed in 5 min at this temperature. However, there is no inactivation of the enzyme at 55 °C if the ionic strength is increased to 1.8 by the addition of NaCl or $(NH_4)_2SO_4$. Since NaCl is known to be an "inert" perturbant of proteins (Von Hippel and Schleich, 1969), ionic strength rather than specific interactions with ions appears to be the major factor in stabilizing the enzyme at the thermophilic growth temperature of 55 °C. However, at 65 °C, stabilization based on the Hofmeister series (Von Hippel and Schleich, 1969) is evident since only 10–20% of the enzymic activity was lost in $(NH_4)_2SO_4$ vs. 60–70% in NaCl. In identical experiments with the crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, increasing the ionic strength had no effect on its thermostability.

The kinetics of inactivation of the thermophilic enzyme in 8 M urea under various conditions are shown in Figure 4. At 30 °C, the rate of inactivation by urea in 0.05 M phosphate buffer (curve 1) is very rapid with a 50% loss in activity ($t_{1/2}$) occurring in 1 min. In a similar experiment with the rabbit

TABLE II: Amino Acid Composition of Glyceraldehyde-3-phosphate Dehydrogenase from *Bacillus coagulans* Grown at 37 and 55 °C.

Amino acid	Amino acid residues per 100-g sample at time of hydrolysis[a] (h)						Std dev	Residues per 144 000*g* protein
	24	24	48	48	72	Av		
Lys	7.02	6.73	5.80	6.29	6.15	6.40	±0.48	79.09
His	3.01	2.86	2.48	2.72	2.57	2.73	±0.21	31.53
Arg	4.57	4.58	4.12	4.12	4.33	4.34	±0.23	44.03
Asx	11.22	11.68		11.14		11.35	±0.29	156.15
Thr	4.66	4.86	3.86	4.84	3.90	5.28[b]		82.69
Ser	4.51	4.76	3.54	3.82	2.90	5.88[b]		106.92
Glx	11.09	11.57		10.66		11.11	±0.46	136.30
Pro	2.51	2.42	2.31		2.52	2.44	±0.32	39.79
Gly	3.84	3.95	3.42	3.56	3.58	3.67	±0.22	101.81
Ala	7.09	7.43	6.27	6.58	6.57	6.79	±0.45	151.30
Val	9.38	9.64	8.66	8.90	9.02	9.12	±0.39	145.75
Met	1.79	1.73	2.16	2.20	1.81	1.94	±0.22	23.41
Ile	4.36	4.42	4.05	4.17	4.22	4.24	±0.22	59.38
Leu	8.20	8.39	7.16	7.44	7.43	7.72	±0.58	108.06
Tyr	3.80	3.96	3.22	3.27	3.35	4.03[b]		29.14
Phe	2.53	2.69	2.21	2.21	2.38	2.40	±0.21	25.86
Trp[c]						0.91	±0.06	7.74
Cys[d]						0.54	±0.03	8.46
Residue weight				90.89				
4 NAD^+ per 146 800*g* enzyme				1.81				
Bound $NH_4^+ + HCO_3^-$				8.05				
Percentage recovery				100.75				

[a] The 24- and 72-h HCl hydrolysates represent enzyme isolated from cells grown at 55 and the 48-h hydrolysates from cells grown at 37 °C. [b] Extrapolated values by least-squares analysis. [c] Determined from 34- and 47-h *p*-toluenesulfonic acid hydrolysates (Liu and Chang, 1971). [d] Determined as cysteic acid from performic acid oxidized enzyme (Hirs, 1956).

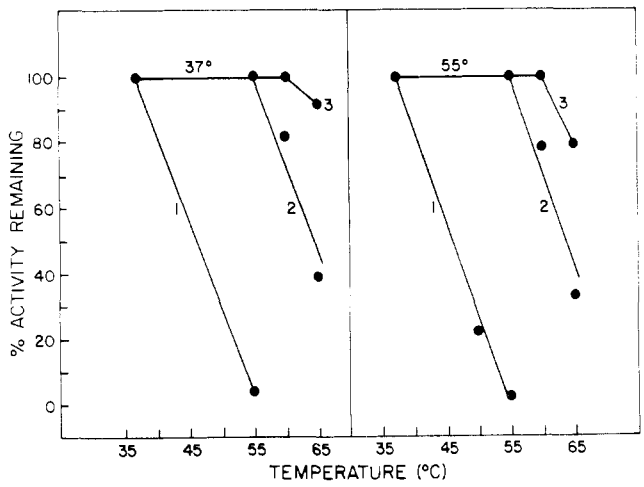

FIGURE 3: Induced thermostability of crystalline glyceraldehyde-3-phosphate dehydrogenase from *Bacillus coagulans* grown at 37 and 55 °C: curve 1, no additions; curve 2, addition of NaCl to an ionic strength of 1.8; curve 3, addition of $(NH_4)_2SO_4$ to an ionic strength of 1.8.

muscle enzyme, a $t_{1/2}$ of less than 0.5 min was observed. These data are consistent with the thermostability studies, and show that the enzyme from *B. coagulans* is much less stable than the enzyme from *B. stearothermophilus* which has a $t_{1/2}$ of 3 h under similar conditions (Amelunxen et al., 1970). In agreement with the thermostability studies, an increase in ionic strength decreases the rate of urea denaturation. However, at 30 °C (curve 3) the rate of inactivation ($t_{1/2}$ = 15 min) is still much greater than that of the enzyme from *B. stearothermophilus*. An increase in ionic strength had very little effect on the rabbit muscle enzyme as the $t_{1/2}$ was less than 1 min. The stabilization of the enzyme from *B. coagulans* by increasing the ionic strength is even more evident at 4 °C where the $t_{1/2}$ went from 13 min (curve 2) to 3.4 h (curve 4).

Discussion

Although glyceraldehyde-3-phosphate dehydrogenase has been purified from numerous sources, this is the first report of crystallization of this enzyme from a facultative thermophile grown at both the mesophilic temperature of 37 °C and the thermophilic temperature of 55 °C. At this stage in our studies, no physicochemical difference has been found in comparative studies of the enzyme obtained from cells grown at both temperatures. These results are in contrast to the report (Balerna and Zuber, 1974) that different types and amounts of purified aminopeptidase are synthesized in an obligate thermophile adapted to grow at both mesophilic and thermophilic temperatures. Other thermoadaptation studies (Jung et al., 1974; Haberstich and Zuber, 1974) have indicated that the level and specific activity of several enzymes from crude extracts (e.g., glyceraldehyde-3-phosphate dehydrogenase) from facultative and obligate thermophiles are markedly different dependent on the growth temperature. As a true facultative thermophile, *B. coagulans* does not require thermoadaptation to grow at the mesophilic and thermophilic growth temperatures. Since the amount, specific activity, and physicochemical properties of the glyceraldehyde-3-phosphate dehydrogenase isolated from *B. coagulans* grown at 37 and 55 °C are essentially identical, there appears to be no relationship to the mechanism involved in the thermoadaptation studies.

The consensus of opinion is that the major mechanism of thermophily in thermophilic bacteria is intrinsic thermostability of the macromolecules, with proteins being studied in most detail (Singleton and Amelunxen, 1973; Williams, 1975; Ljungdahl and Sherod, 1976). For example, homogeneous glyceraldehyde-3-phosphate dehydrogenase from *B. stearothermophilus* 1503 with bound NAD^+ (Amelunxen, 1966) or as the apoenzyme (Amelunxen and Clark, 1970) can withstand temperatures greater than 80 °C in deionized water without loss of activity. In sharp contrast to this is the essentially complete inactivation of the enzyme from *B. coagulans*

when heated for 5 min at 55 °C (thermophilic growth temperature) in 0.05 M phosphate buffer; even the mesophilic sources of this enzyme, e.g. from *B. cereus* (Suzuki and Imahori, 1973) and rabbit muscle (Amelunxen, 1966), under similar conditions retain greater than 90 and 50% activity, respectively.

This is the first well-documented report of a homogeneous thermolabile enzyme from a thermophilic organism. Since Novitsky et al. (1974) noted that several of the glycolytic enzymes that were assayed in crude extracts of *B. coagulans* were thermolabile, this may be a general feature of enzymes from this organism. Two possible explanations of how this enzyme can function in vivo are rapid resynthesis of the enzyme or intracellular stabilizing factors. Rapid resynthesis does not appear to be likely since the generation time is very similar at both growth temperatures (Novitsky et al., 1974; Crabb et al., 1975), and there is no evidence for such a mechanism in studies with other thermophiles (Singleton and Amelunxen, 1973). Although studies of thermophilic proteins have contraindicated the necessity for nonspecific protective factors (Singleton and Amelunxen, 1973) such a mechanism seems feasible in considering the stabilization in vivo of glyceraldehyde-3-phosphate dehydrogenase from *B. coagulans*.

The observation that 10% but not 2% ammonium sulfate stabilizes the glyceraldehyde-3-phosphate dehydrogenase from *B. coagulans* in the cold led to the discovery that increasing the ionic strength by a factor of 1.8 would protect the enzyme against thermal inactivation in crude extracts (Crabb et al., 1975). In order to rule out the effect of other factors in the crude extracts, these measurements were repeated on the homogeneous enzyme (Figure 3) with similar results. Preliminary experiments (unpublished) concerned with titrating the ionic strength effect have shown that maximal protection is conferred to the homogeneous enzyme by bringing the ionic strength to 1.8 (using NaCl). Since divalent anions such as sulfate and phosphate are known protein stabilizers, and one of the substrates for this enzyme is phosphate, sodium chloride was used in these experiments to rule out the necessity for divalent anions in conferring thermostability. Although phosphate does provide thermal protection at higher concentrations, the concentration used in these studies (0.05 M) does not, and this latter value is five times higher than the intracellular concentration reported by Damadian (1973) for *Escherichia coli*. Even at the intracellular neutral salt concentration of approximately 0.3 M (Damadian, 1973), there is extensive inactivation of the enzyme. The thermolability of the glyceraldehyde-3-phosphate dehydrogenase in crude extracts (Crabb et al., 1975) at the protein concentrations used would seem to eliminate specific binding of other cellular components (e.g., substrates and coenzymes) that could stabilize the enzyme. The fact that the homogeneous enzyme is equally thermolabile to that in crude extracts seems to rule out the presence of destabilizing factors in the crude extracts. Hence, the stabilization of this enzyme in vivo must be dependent upon the intracellular concentration of other components. Since the enzyme is stabilized at a fairly high ionic strength, and Damadian (1973) has shown that the concentration of ionic groups in the bacterial cell is equivalent to 1 *m* (80% due to macromolecules) we postulated that the intracellular concentration of ionic groups confers thermostability to the enzyme in vivo (Crabb et al., 1975). Although further studies are needed to substantiate this proposal, it nevertheless seems to be the best explanation for the in vivo stabilization of this enzyme, and the data in this paper concerned with the homogeneous enzyme are consistent with our previous observations.

In the initial studies to determine the structure-function

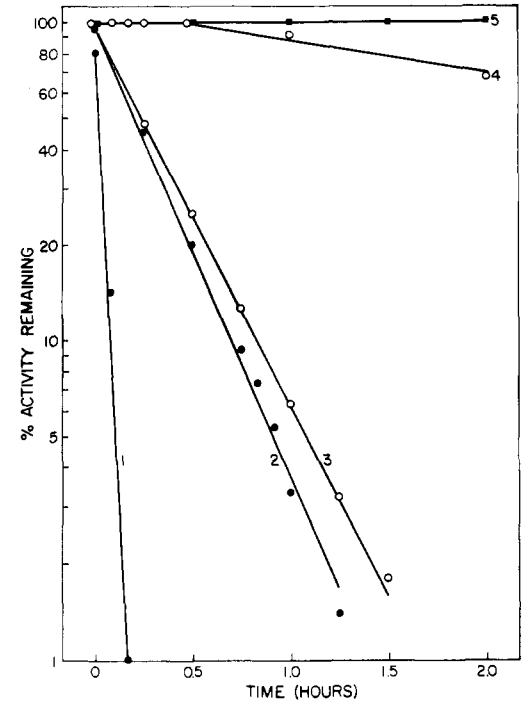

FIGURE 4: Rate of inactivation of crystalline glyceraldehyde-3-phosphate dehydrogenase from *Bacillus coagulans* (grown at 55 °C) in 8 M urea under different solvent and temperature conditions: curve 1, incubation at 30 °C and pH 7.1 in 8 M urea-0.05 M sodium phosphate buffer-10^{-3} M EDTA; curve 2, incubation at 4 °C in the solvent above; curve 3, incubation at 30 °C and pH 7.1 in 8 M urea-0.05 M sodium phosphate buffer-10^{-3} M EDTA-10% NaCl; curve 4, incubation at 4 °C in solvent directly above; curve 5, composite of controls without urea from all systems above.

relationship to thermostability, some of the physicochemical properties of glyceraldehyde-3-phosphate dehydrogenase were determined. Typical of this enzyme from other sources, it is a tetramer with the same sedimentation rate and contains firmly bound NAD^+, which suggests that its unusual thermolability is not due to gross size and conformational differences. Also typical of this enzyme from other sources, there is no detectable amount of other components bound to the enzyme as evidenced by quantitative recovery of the sample based on the amino acid analysis and NAD^+ content (Table II). The thermal inactivation of the enzyme does not appear to be due to oxidation of the sulfhydryl groups since DDT and EDTA were both present, and enzyme activity could not be restored by preincubation with cysteine. The loss of bound NAD^+ and the relationship to thermostability have also been considered. A repeat of the heat treatment experiments at 55 °C with subsequent dialysis to remove any free NAD^+ and measurement of the A_{280}:A_{260} ratio indicated that the samples with and without NaCl still contained the full complement of bound NAD^+. The urea denaturation studies showed that the enzyme from *B. coagulans* is more resistant to denaturation than the muscle enzyme, but is inactivated at a significantly faster rate than the enzyme from *B. stearothermophilus* (Amelunxen et al., 1970). The enhanced resistance to urea denaturation by increasing the ionic strength to 1.8 is good evidence that the thermal stabilization is related to the conformational stability of the enzyme, rather than to a minor change such as the oxidation of sulfhydryl groups. Detailed studies on the conformational changes and function of this enzyme at different temperatures and ionic strengths should determine the degree of unfolding associated with the thermal inactivation.

Recently, the three-dimensional structure of glyceralde-

TABLE III: Amino Acid Composition of the Subunit of Nine[a] Glyceraldehyde-3-phosphate Dehydrogenases.

Amino acid	Thermophilic				Mesophilic				
	T.t.	T.a.[b]	B.s.[b]	B.co.	B.c.	E.c.	y[b]	L.m.[b]	P.m.[b]
Lys	19	22	23	20	25	26	26	28	26
His	8	10	9	8	7	6	7	5	11
Arg	17	16	14	11	13	12	11	9	10
	(44)	(48)	(46)	(39)	(45)	(44)	(44)	(42)	(47)
Asx	31	34	40	39	40	45	37	32	38
Glx	26	23	26	34	27	22	23	24	18
	(57)	(57)	(66)	(73)	(67)	(67)	(60)	(56)	(56)
Gly	37	25	25	25	32	31	29	30	32
Ser	12	13	17	27	14	14	24	25	19
Pro	6	12	11	10	10	9	13	12	12
	(55)	(50)	(53)	(62)	(56)	(54)	(66)	(67)	(63)
Thr	23	22	18	21	24	28	23	20	22
Cys	1	1	2	2	2	4	2	5	4
	(24)	(23)	(20)	(23)	(26)	(32)	(25)	(25)	(26)
Ala	39	42	39	38	36	35	33	32	32
Val	30	30	43	36	32	32	36	38	34
Met	5	7	7	6	7	8	6	10	9
Ile	18	22	19	15	21	17	19	18	21
Leu	33	31	26	27	21	20	22	18	18
Tyr	9	10	7	10	7	8	10	9	9
Phe	7	7	5	6	12	11	10	15	14
Trp	N.a.	3	2	2	N.a.	4	3	3	3
	(141)	(152)	(148)	(140)	(136)	(135)	(139)	(143)	(140)
Total residues	321	332[c]	333	337	330	332	334	333	332

[a] Abbreviations and references are: T.t. = *Thermus thermophilus* (Fujita et al., 1976); T.a. = *Thermus aquaticus* (Hocking and Harris, 1976); B.s. = *Bacillus stearothermophilus* (Biesecker et al., 1977); B.co. = *Bacillus coagulans* KU; B.c. = *Bacillus cereus* (Suzuki and Imahori, 1973); E.c. = *Escherichia coli* (D'Alessio and Josse, 1971); y = yeast (Jones and Harris, 1972); L.m. = lobster muscle (Davidson et al., 1967); P.m. = pig muscle (Harris and Perham, 1968); N.a. = not available. [b] From amino acid sequence data. [c] Two residues in the sequence were not identified (positions 303 and 304).

hyde-3-phosphate dehydrogenase from *B. stearothermophilus* has been reported (Biesecker et al., 1977), but no unequivocal molecular reasons for the remarkable thermostability of this enzyme were apparent. However, the differences in thermostability of homologous enzymes from different sources must reside in the amino acid composition and sequence. The unexpected thermolability of the glyceraldehyde-3-phosphate dehydrogenase from *B. coagulans* provides a unique opportunity to investigate differences in the amino acid content and sequence that might account for variations in the thermostability of proteins. Although there is a great deal of similarity in the amino acid composition (Table III) there are some interesting differences. The cysteine content is probably not related to thermostability since the enzyme from mesophilic and thermophilic bacilli contains two residues per subunit. The enhanced thermostability due to ionic strength could be related to an increase in hydrophobic interactions, but the similarity in the content of hydrophobic residues in all sources of the enzyme would suggest that this is not the case. From Table III, the serine content is higher than that found in the enzyme from other thermophilic sources. As noted with ribonuclease A (Burgess and Scheraga, 1975), a segment containing a high serine content may be thermolabile. However, stabilization by increasing the ionic strength would not be expected to occur if serine were the only residue involved. It is also apparent that the basic amino acid content of the enzyme from *B. coagulans* is significantly lower than that found in counterparts from other sources. Although this may be related to the thermolability of the enzyme, a decrease in the basic residues could not explain the ionic strength effect. There is also a higher level of glutamate–glutamine than that found in either mesophilic or thermophilic sources. Isoelectric focusing experiments in 8 M urea indicated that the enzyme from *B. coagulans* has a p*I* less than the value of 4.6 reported for this enzyme from *B. stearothermophilus* (Singleton et al., 1969), indicating that only a few of the residues could be glutamine. The apparent increase in glutamic acid residues may be related to the difference in thermostability at different ionic strengths. For example, two or more acidic residues in the same vicinity of the molecule could destabilize the enzyme by electrostatic repulsion, whereas an increase in ionic strength would overcome this repulsion. The secondary structure associated with the region of substitution could also be an important factor. For example, Chou and Fasman (1974) have found that glutamic acid is very frequently located in helical regions but is not likely to occur in β structure. Critical amino acid substitutions are undoubtedly a major consideration in controlling the stability of protein molecules, and are probably the key to understanding the molecular reasons for divergent thermophilic mechanisms.

It is apparent that the molecular reasons for the unusual thermolability of glyceraldehyde-3-phosphate dehydrogenase from *B. coagulans* are as evasive and intriguing as those involved in conferring intrinsic thermostability to this enzyme from obligate and caldoactive thermophiles. However, a novel feature of the enzyme from the facultative thermophile is that it can readily be converted from a thermolabile to a thermostable state, which provides an unusual system for uncovering new information on thermophilic mechanisms and protein structure.

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