A PROPOSED MECHANISM OF THERMOPHILY IN FACULTATIVE THERMOPHILES

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Glyceraldehyde-3-phosphate dehydrogenase in crude extracts of Bacillus coagulans KU, a facultative thermophile, showed marked thermolability whether the cells were grown at mesophilic or thermophilic temperature. When extracts were brought to a given ionic strength by the addition of salt and then heat treated, it was possible to confer heat resistance well in excess of the thermophilic growth temperature. Disc electrophoresis is indicative that portions of the profiles are quite different between extracts of cells grown at 37° and 55°. Based on the data, a mechanism of thermophily is postulated that is different from any thus far proposed for thermophilic microorganisms.

INTRODUCTION

There is little supportive evidence for any explanation of thermophily except the hypothesis that proteins from obligate thermophiles are intrinsically thermostable (1). Extensive physicochemical characterization of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from B. stearothermophilus 1503 in this laboratory (1) lends support to this hypothesis. Studies with crude extracts of B. stearothermophilus and B. cereus (2) showed the marked thermostability of eleven enzymes from the thermophile, in contrast to the thermolability of the mesophilic counterparts. Novitsky et al. (3) in studies with the cell walls of B. coagulans KU reported that selected glycolytic enzymes from crude extracts of cells grown at 37° and 55° are as labile as those from mesophilic organisms, suggesting that enzymes from facultative thermophiles do not exhibit intrinsic thermostability. They concluded that differences in composition between the cell walls of this organism grown at both temperatures could not explain the enhanced thermostability at 55°, and proposed that an increased rate of protein synthesis may allow these organisms to grow at elevated temperatures.

In the course of experiments to purify glyceraldehyde-3-phosphate dehydrogenase from B. coagulans KU grown at both 37° and 55°, a marked lability

of the enzyme was noted in buffers of low ionic strength. Also, the enzyme was heat inactivated well below the thermophilic growth temperature in extracts of cells grown at either temperature. On the basis of induced thermostability of the enzyme by increasing the ionic strength, and a marked resistance to heat denaturation of the proteins in extracts of cells grown at 55°, a mechanism of thermophily for facultative thermophiles is proposed.

MATERIALS AND METHODS

The bacterium B. coagulans KU was grown in a 30 liter Fermentation Design fermentor in Difco antibiotic medium 3 (supplemented with 0.2% glucose) with vigorous aeration at both 37° and 55°. Under these conditions, cells grown at 37° and 55° reached the end of log phase at 2.7h and 2h respectively. Cells in the log phase were cooled immediately, centrifuged in a Sorvall continuous flow centrifuge at 27,000 X g, washed in 0.15 M NaCl, and resuspended in a minimal volume of deionized water containing 10^{-3}M EDTA and dithiothreitol to protect the sulfhydryl groups of the enzyme. The cells were then ruptured in a Branson Sonifier (Model S-125) for 5 min.

Crude extracts (approximately 25 mg protein per ml) of cells grown at 37° and 55° were divided into portions, and the ionic strength was increased by a factor of 1.8; this corresponded to the addition (in solid form) of 8% $(NH_4)_2SO_4$ and 10% NaCl. Following heat treatment for 5 min at the temperatures indicated in Figs. 1 and 3, the samples were cooled rapidly, centrifuged, and the supernatant fluids assayed for glyceraldehyde-3-phosphate dehydrogenase (4). The optimal pH for enzymic activity was found to be 8.6 for crude extracts or for partially purified enzyme. Crude or purified enzyme from extracts of cells grown at either temperature is fully active without cysteine. However, because of rapid inactivation of the enzyme on dilution in the assay system, cysteine was routinely included. Protein determinations were carried out using a semi-micro biuret method (5). Disc electrophoresis of crude extracts of B. coagulans grown at both 37° and 55° was carried out essentially as described by Ornstein and Davis (6).

RESULTS

Heat denaturation.[1] A major difference between cells grown at 37° and 55° is the complete resistance to heat denaturation of the proteins in extracts of cells grown at the thermophilic temperature (Fig. 1). The addition of salts to the extracts in Fig. 1B had little effect on protein denaturation, in contrast to the marked effect on extracts in Fig. 1A. Even under these conditions, more protein remained in solution in the extracts of cells grown at 55°. Polyacrylamide gel electrophoresis (Fig. 2) provides additional evidence for a major difference in the protein population of these extracts. Several additional slower moving components are present in extracts of cells grown at 55°, whereas the faster moving components in the lower half of the gels looked similar. Consistent with this observation is the presence of a greater amount of high molecular weight components in extracts of cells

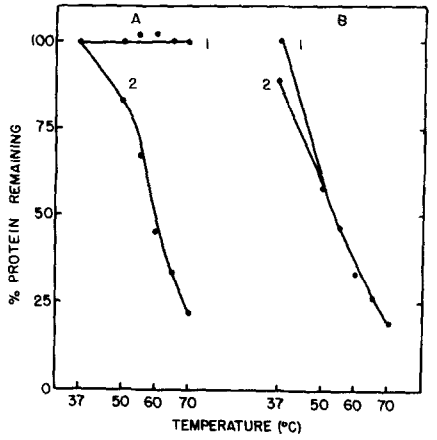

Fig. 1. Heat denaturation of proteins from extracts of Bacillus coagulans grown at 55° (A) and 37° (B). Experimental procedures as described in Materials and Methods. Curve 1, no addition of salt; curve 2, addition of NaCl or $(NH_4)_2SO_4$; a composite curve was drawn since differences in the salt effects were not significant. Controls for each of the above were kept at 4° throughout the experiment; there was no change in the protein content. In other experiments, heat treatment at 55° and 60° was extended to 15 min for A and B; there was no significant change in the protein content in either system.

1 Heat denaturation is defined here as a major alteration as evidenced by precipitation, and heat inactivation as a loss of function without precipitation.

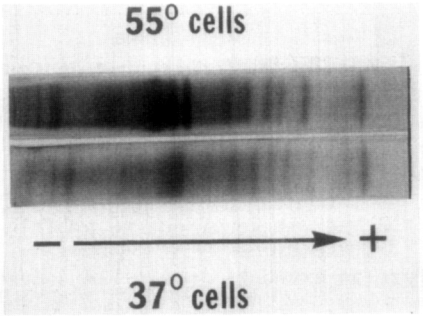

Fig. 2. Electrophoretic profiles of extracts of Bacillus coagulans grown at 37° and 55°. Each gel contained approximately 200µg protein.

grown at the thermophilic temperature during a purification step using molecular sieve chromatography.

Properties of glyceraldehyde-3-phosphate dehydrogenase from cells grown at 37° and 55°. Partially purified enzyme has been obtained from cells grown at both temperatures. The amount and specific activity are essentially the same at each step of purification. The purified enzyme in either extract is extensively inactivated at low ionic strength after 24h at 4°, but there is little inactivation when the ionic strength is increased by a factor of 1.8. Based on molecular sieve chromatography and fractionation with $(NH_4)_2SO_4$, the enzyme from both extracts shows partial dissociation at high salt concentrations.

Induced thermostability of glyceraldehyde-3-phosphate dehydrogenase. By increasing the ionic strength of extracts of cells grown at 37° and 55°, thermostability is induced to the enzyme significantly beyond the thermophilic growth temperature, whereas extensive heat inactivation occurs below this temperature in the absence of added salts (Fig. 3). Since heat denaturation of proteins does not occur in extracts of cells grown at 55° (Fig. 1A), the heat inactivation of the enzyme (Fig. 3A) probably is not due to major structural changes. Hence the enzyme may have inherent resistance to heat denaturation but is subject to heat inactivation in an environ-

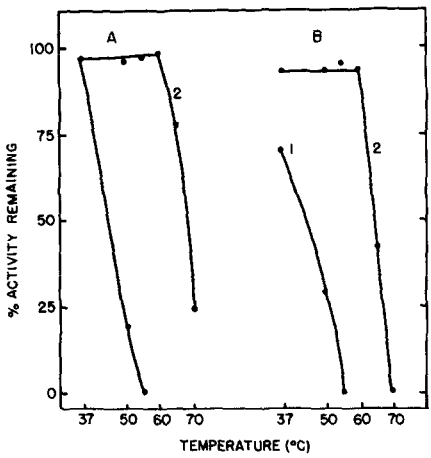

Fig. 3. Induced thermostability of glyceraldehyde-3-phosphate dehydrogenase from extracts of Bacillus coagulans grown at 55° (A) and 37° (B). Experimental procedures as described in Materials and Methods. Curve 1, no addition of salt; curve 2, addition of NaCl or $(NH_4)_2SO_4$; a composite curve was drawn since differences in the salt effects were not significant. Controls for each of the samples above were kept at 4° throughout the experiment; there was no change in the enzymic activity. In other experiments, heat treatment at 55° and 60° was extended to 15 min for A and B; there was no significant additional loss in enzymic activity in either system.

ment of low ionic strength. In addition, the enzyme shows slightly more stability in extracts of cells grown at 55° with salt added, than in extracts of cells grown at the mesophilic temperature. This seems to parallel the greater resistance to heat denaturation of the proteins in extracts of cells grown at 55°, and suggests that the proteins in the extracts are providing a stabilizing effect.

DISCUSSION

A substantial amount of data has accumulated verifying that a primary mechanism of thermophily for obligate thermophiles involves inherent thermostability of the macromolecules (1). However, for facultative thermophiles, e. g. B. coagulans KU, intrinsic molecular thermostability does not appear to be the case. The evidence presented in this paper and by other investigators (3) is indicative that enzymes from this organism are as labile (or more so) than their counterparts from mesophilic sources.

Although an increased rate of protein biosynthesis could be a factor in maintaining enzyme activity in the cell, the growth rate of B. coagulans at

37° and 55° as reported previously (3) and confirmed in our laboratory is much closer than would be expected if this were the mechanism. Brock (7) has compiled the optimal growth temperatures and growth rates for thermophilic and mesophilic organisms and plotted the data in a double reciprocal Arrhenius manner. The plots strongly suggested that thermophiles do not grow as rapidly as would be predicted from an Arrhenius relationship; it was concluded that survival at elevated temperatures is not simply a function of rapid resynthesis of heat-denatured cellular components.

How facultative thermophiles survive at elevated temperatures presents therefore a most perplexing question. The effect of ions and other small molecules on the catalytic environment has been reviewed extensively by Grisolia (8), but little information is available that is directly pertinent to the studies presented in this paper. Based on the experiments reported here, it is suggested that these organisms adapt to higher growth temperatures by establishing a more stable macromolecular environment that can induce heat stability to enzymes that are thermolabile when removed from the intracellular milieu. Such a mechanism would require much smaller alterations in the cellular metabolism than specific alterations of each enzyme. The resistance to heat denaturation of extracts of cells grown at 55° shows that B. coagulans modifies the environment such that the proteins are not subject to heat denaturation at the thermophilic growth temperature. The disc electrophoresis patterns suggest that a different population of proteins exists in these extracts. Experiments in progress are designed to assess whether this change represents either new types of protein being synthesized or modification of existing heat denaturable proteins by attachment of other components such as carbohydrate moieties. Of much interest in this regard are the unique glycoproteins found in antarctic fish that allow them to live at subzero temperatures (9).

Since the enzymes extracted from the facultative thermophile, B. coagulans KU, do not have inherent thermostability, the intracellular environment

must be providing this heat resistance. The induced thermostability of glyceraldehyde-3-phosphate dehydrogenase upon increasing the ionic strength of extracts of cells grown at 37° and 55° shows that a highly charged environment can afford this stability very effectively. Although it is speculative to assume that cells grown at 37° and 55° would contain salt concentrations at the level used in this study, the extensive work of Damadian (10) has shown that the ionic environment of Escherichia coli is 1 molal which is similar to the salt concentrations used in this study.

Based on these data and the growing realization that the microbial cell is highly organized with little intracellular free water, the facultative thermophile can be visualized as containing a highly charged macromolecular environment that can induce thermostability to proteins that otherwise would be heat inactivated, and can modify other proteins to prevent heat denaturation at the thermophilic growth temperature.

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