Biochimica et Biophysica Acta, 452 (1976) 580—596

BBA 67990

DIHYDRODIPICOLINIC ACID SYNTHASE OF *BACILLUS LICHENIFORMIS*

QUATERNARY STRUCTURE, KINETICS, AND STABILITY IN THE PRESENCE OF SODIUM CHLORIDE AND SUBSTRATES

S.M. HALLING * and D.P. STAHLY

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242 (U.S.A.)

(Received June 9th, 1976)

Summary

Dihydrodipicolinic acid synthase (L-aspartate-β-semialdehyde hydro-lyase (adding pyruvate and cyclising), EC 4.2.1.52) obtained from *Bacillus licheniformis* was purified to homogeneity. Its molecular weight was 108 000 to 117 500, depending on the concentration of NaCl and substrates present, and it contained four subunits of identical molecular weight (28 000). The K_m values for pyruvate and L-aspartic semialdehyde were approximately 5.3 and 2.6 mM, respectively. It was previously shown that pyruvate and a high sodium chloride concentration contributed to the stability of the enzyme. The effect of these substances and the other substrate, L-aspartic semialdehyde, on molecular weight was determined. None of these three substances significantly affected the apparent molecular weight. The effect of sodium chloride, pyruvate, and L-aspartic semialdehyde on enzyme structure was studied by determining the effect of their presence on inactivation of the enzyme by several chemical denaturants and heat. Pyruvate dramatically protected against inactivation by all of the denaturants. Sodium chloride protected against inactivation by sodium dodecyl sulfate, guanidine · HCl, urea, and heat, but somewhat facilitated inactivation by ethanol. L-Aspartic semialdehyde had no significant effect on inactivation by sodium dodecyl sulfate and ethanol; it rendered the enzyme slightly more sensitive to inactivation by guanidine · HCl and urea. The thermal melting curve obtained for the enzyme in the presence of L-aspartic semialdehyde was biphasic. The activity was reduced approximately 50% by heating for 30 min at temperatures between 50 and 80°C. Only by heating at temperatures above 80°C did the inactivation become complete. The partially inactivated enzyme could be reactivated by heating after removal of the L-aspartic semialdehyde. Pyruvate prevented the partial

* Present address: Department of Biochemistry and Biophysics, University of California, Davis, Calif. 95616 (U.S.A.)

inactivation and facilitated reactivation. The only difference detected between the native enzyme and the partially inactivated form of the enzyme was that the latter had a reduced V. It is known that in other spore-formers, dihydrodipicolinate synthase increases in activity late in sporulation. This increase may be important for normal sporulation to occur. The possibility is discussed that the intracellular pool sizes of pyruvate and L-aspartic semialdehyde might have an influence on the level of dihydrodipicolinate synthase activity, by controlling the amount of partial inactivation of the enzyme that occurs in vivo.

Introduction

Dihydrodipicolinic acid synthase (L-aspartate-β-semialdehyde hydro-lyase (adding pyruvate and cyclising), EC 4.2.1.52) catalyzes the direct condensation of pyruvic acid and L-aspartic β-semialdehyde to form dihydrodipicolinic acid, an intermediate in the biosynthesis of diaminopimelic acid and lysine. In bacteria forming endospores, dihydrodipicolinic acid is also converted to dipicolinic acid, which accumulates in large quantities in endospores [1]. Dihydrodipicolinate synthase in spore-formers is unusual in that it does not seem to be regulated by inhibition, activation, repression or induction [2—8].

Dihydrodipicolinate synthase of *Bacillus licheniformis* was partially purified by Stahly [4]. The enzyme required 2 M NaCl for stability. Also, in the course of purification, it was discovered that pyruvate dramatically stabilized the enzyme against heat-inactivation. Pyruvate also enhances thermal stability of dihydrodipicolinate synthase from *Bacillus megaterium* [5]. As substrates and NaCl sometimes stabilize enzymes by causing association or dissociation of subunits, the molecular weight and subunit structure of dihydrodipicolinate synthase was determined, and the effect of substrates and NaCl on quaternary structure was studied. It was found that the enzyme has four subunits of the same molecular weight. Pyruvate, L-aspartic semialdehyde, and NaCl had no effect on quaternary structure. Pyruvate and NaCl apparently stabilize the molecule by causing more subtle changes in structure of the enzyme, as demonstrated herein by their effects on inactivation by a variety of chemical denaturants and heat. The most unusual finding of this study was that in the presence of L-aspartic semialdehyde, dihydrodipicolinate synthase exhibited a biphasic melting curve. Partial inactivation of the enzyme to approximately 50% of the original activity occurred by heating for 30 min at temperatures between 50 and 80° C. Complete inactivation occurred at temperatures above 80° C. Pyruvate protected against this L-aspartic semialdehyde facilitated inactivation. This partial inactivation of the enzyme in the presence of aspartic semialdehyde at rather low temperatures was found to be the cause of the sigmoid shape of the previously observed dihydrodipicolinate synthase-pyruvate saturation curve [4]. It is possible that this phenomenon of partial inactivation might be the basis of a novel control mechanism.

Materials and Methods

Culturing and harvest of the microorganism

B. licheniformis A-5 was grown, as previously described [4], in an am-

monium lactate/glucose/salts medium, and was harvested in the late exponential phase of growth when the culture turbidity was 230 Klett Units (as measured by a Klett-Summerson photoelectric colorimeter with a number 54 filter). The cell pellet, obtained by centrifugation of the cells, was washed twice in buffer (2 M NaCl, 0.05 M sodium barbital; pH 8.0) and then was frozen at 20° C until needed.

Preparation of cell extracts

Cells were suspended in the buffer described above and were broken by use of a modified [9] French Pressure Cell (American Instrument Co., Inc., Silver Spring, Maryland). The whole cells and cell debris were removed by centrifugation, and the extract was dialyzed overnight against the same buffer. The extract was then stored at 0° C. Dihydrodipicolinate synthase was stable under these conditions for at least 3 months.

Protein determination

Initial cell extracts and enzyme preparations purified through the heat step (see Table I) were assayed for protein concentration by the biuret method [10]. The protein concentration at other stages of purification was determined by the Lowry technique [11]. Protein concentration of fractions eluted from Sephadex G-200 and Diethylaminoethyl (DEAE)-Sephadex columns was estimated using the absorbance at 280 nm or the Lowry technique. Bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) was used as a standard.

Synthesis and assay of L-aspartic semialdehyde

The procedures used for synthesis and assay of L-aspartic semialdehyde were described previously [7].

Assay of dihydrodipicolinate synthase

Dihydrodipicolinate synthase was assayed by the *o*-aminobenzaldehyde method as described previously [7]. Unless otherwise specified, the L-aspartic semialdehyde and pyruvate concentrations were 9 mM and 20 mM, respectively. One unit of enzyme is defined as that catalyzing an increase of 1.0 in absorbancy at 520 nm per min at 37° C. Specific activity is defined as units of enzyme per mg of protein. A linear relationship was found between A_{520} and reaction time, and between A_{520} and amount of enzyme, when the A_{520} did not exceed 0.7.

Purification of dihydrodipicolinate synthase

Purification steps were carried out at 0—4° C unless noted otherwise. The steps are as described below.

Step 1. The cell extract was prepared as described above.

Step 2. Fractionation by heat. The cell extract from step 1 was dialyzed overnight against 2 M NaCl, 0.05 M sodium barbital buffer (pH 8.0), and then adjusted to pH 8.5 by the addition of a small volume of 2 M Tris. This pH was found to be optimal for heat stability. The extract was heated at 70° C for 10 min. The denatured protein was removed by centrifugation at 25 000 × *g* for 15 min.

Step 3. Ethanol fractionation. A final concentration of 35% ethanol was achieved by the addition of 95% ethanol. The ethanol, which had been cooled to 0°C, was added rapidly with constant stirring. This procedure was used to precipitate extraneous proteins which were subsequently removed by centrifugation at 25 000 × *g* for 10 min.

Step 4. Streptomycin sulfate treatment. Before addition of the streptomycin sulfate, the ethanol was removed and the ionic strength was decreased by dialysis of the supernatant fluid from step 3 against 0.005 M sodium barbital buffer, 0.005 M potassium pyruvate (pH 8.0). A 1:1 ratio (w/w) of streptomycin sulfate to protein was found to be optimal. The streptomycin sulfate (10% aqueous solution, pH 7.5) was added slowly with stirring, and stirring was continued for 20 min after the addition was complete. The precipitate was removed by centrifugation at 25 000 × *g* for 10 min.

Step 5. Ammonium sulfate fractionation. The supernatant fluid from step 4 was dialyzed against a buffer containing 0.05 M sodium barbital, 2 M NaCl (pH 8.0) before addition of ammonium sulfate. During the fractionation, the temperature was maintained at 0°C, and the pH was maintained above 7.7 by the addition of concentrated ammonium hydroxide. Four sequential additions of ammonium sulfate were made routinely; 33, 7.4, 8.7 and 7.4 g, all per 100 ml of extract. The precipitate was removed by centrifugation between additions. Each of the pellets was extracted repeatedly with the buffer referred to above. All of the enzyme went into solution in this buffer, whereas much of the other protein remained insoluble. Usually only the third fraction had a high specific activity, but some variation occurred between enzyme preparations. When more than one fraction exhibited a high specific activity, the fractions were combined.

Step 6. Gel exclusion chromatography. The supernatant fluid from step 5 was dialyzed against 1 M NaCl, 0.05 M sodium barbital (pH 8.0) and was concentrated to 8 ml by use of a 25 ml ultrafiltration cell (Amicon Corporation, Lexington, Massachusetts) with a type UM-10 filter. A 2.5 cm × 100 cm column containing 88 cm bed height of Sephadex G-200 gel (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey) was used. The buffer used for column equilibration and elution was the same as above. Ascending chromatography was employed, solvent flow being maintained by gravity feed. A single peak of enzyme activity was obtained, the most active fraction being at an elution volume of 186 ml. The fractions with high specific activity were combined.

Step 7. DEAE-Sephadex column chromatography. The enzyme was eluted from a 2.5 × 125 cm column containing DEAE-Sephadex, type A-25-120. A buffer containing 0.005 M sodium barbital, 0.05 M NaCl, and 0.005 M potassium pyruvate (pH 8.0) was used for dialysis of the combined fractions from step 6 and for equilibration of the column. After application of the enzyme to the column, it was eluted with a linear NaCl gradient generated by use of two reservoirs, one containing 1 liter of equilibration buffer, and the other, 1 liter of a similar buffer with 0.5 M NaCl added. The molarity of the NaCl in the fractions was determined by use of a conductivity meter (Radiometer Company, Copenhagen). The NaCl concentration in the fraction containing the highest activity was 0.18 M. The specific activity of each of the fractions containing high activity was determined. Usually the specific activity of these frac-

tions was identical. If the specific activities varied, the active fractions were rechromatographed on DEAE-Sephadex. The active fractions were combined and stored at 2—4° C.

Molecular weight determination

Gel filtration as described by Andrews [12] was used to determine the molecular weight of dihydrodipicolinate synthase. A 2.5 × 45 cm column was prepared with a 36 cm bed height of Sephadex G-200 or Biogel P-200 (Bio-Rad Laboratories, Richmond, California). The column was equilibrated at 4° C with 2 bed volumes of the elution buffer (see Results). Ascending chromatography was employed, solvent flow being maintained by gravity feed. The void volume was determined by use of Dextran Blue 2000 (Pharmacia Fine Chemicals, Inc.). The enzyme and several standard proteins were chromatographed, and the K_{av} for each was determined. Protein standards used included bovine γ-globulin (160 000), rabbit muscle aldolase (158 000), bovine serum albumin (68 000), ovalbumin (45 000), and chymotrypsinogen A (25 000). The molecular weight of the enzyme was determined from a column calibration curve consisting of a plot of the K_{av} values of the standard proteins versus the $\log_{10}$ of their molecular weights.

Polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was conducted by the method of Davis [13] with a Buchler Polyanalyst Electrophoresis Apparatus and a Buchler Power Supply No. 3-1014A (Buchler Instruments, Fort Lee, New Jersey). Purified enzyme was mixed with an equal volume of 20% glycerol and layered above a spacer gel in glass tubes (0.6 × 10 cm). After electrophoresis, the gels were removed from the tubes, severed with a scalpel at the leading edge of the tracking dye (bromphenol blue), and then the gels were either stained for protein with Xylene Brilliant G (Brilliant Blue G; Sandoz Colors and Chemicals, Chicago, Illinois) at 65° C for 30 min, or for enzymatic activity. The protein staining solution was prepared by dissolving 1.25 g of dye in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid and filtering to remove insoluble material. After the stained gels were rinsed in water, the gels were destained at 65° C with several changes of a solution containing methanol/acetic acid/water (2:3:3.5, by vol.) [14]. Enzymatic activity was localized by placing the gels in double strength reaction mixture and incubating at 37° C until a faint colored band appeared (about 1 h). The reaction mixture was then replaced with stopping buffer to enhance the color of the band.

Subunit molecular weight determination

The subunit molecular weight of purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described by Laemmli [15], with the following modifications. The denaturation of the enzyme and protein standards was conducted at 100° C for 5 min instead of 1.5 min. No spacer gel was used. The current was kept constant at 8 mA per gel. After electrophoresis the gels were stained with Xylene Brilliant G. The protein standards of known subunit molecular weight were myoglobin (17 200),

trypsin (22 300), glyceraldehyde-3-phosphate dehydrogenase (37 000), aldolase (40 000), ovalbumin (45 000) and bovine serum albumin (68 000). A plot of the $\log_{10}$ of the subunit molecular weights of the standard proteins versus their relative mobility provided a standard curve [14].

Procedure for heating dihydrodipicolinate synthase and for analysis of results

In all cases the heating medium included 0.05 M sodium barbital buffer (pH 8.5). Different concentrations of NaCl, pyruvate, and L-aspartic semialdehyde were included, as described in Results. Portions of the enzyme (0.25 to 0.5 ml) were placed in 10 × 100 mm screw-cap tubes. The tubes were placed in a water bath at the appropriate temperature, and individual tubes were removed at specified times and placed on ice. The enzyme samples were then assayed for enzymatic activity.

The activation energy (E_a) for denaturation was determined by use of the Arrhenius equation ($k = Ae^{-E_a/RT}$) and the observed thermal inactivation rate constants (k values) obtained by heating the enzyme at different temperatures. Other thermodynamic constants were determined assuming thermal inactivation to be an activation process, and, thus, amenable to treatment according to the Eyring theory of absolute reaction rates [16,17]. The free energy change due to the formation of the activated complex, $\Delta F^{\ddagger}$, was determined from the relationship, $\Delta F^{\ddagger} = -RT \ln (kh/K_b t)$. The inactivation rate constant (k) for this calculation was determined by heating at 74°C. The enthalpy change due to the formation of the activated complex, $\Delta H^{\ddagger}$, was determined from the relationship, $\Delta H^{\ddagger} = E_a - RT$. The entropy change due to the formation of the activated complex, $\Delta S^{\ddagger}$, was calculated from the relationship, $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$.

Statistical analysis of data

Least-squares linear regression was used to determine the straight line that best fit the data points, on data graphically portrayed as a straight line. The "degree of fit" of the data points to the least-squares straight line was determined by calculating the correlation coefficient, r. The r values are given in the figure legends.

Results

Dihydrodipicolinate synthase obtained from exponential phase cells of *B. licheniformis* was purified 766-fold (Table I). The evidence for purity of the enzyme was as follows. (i) The enzyme in the separate fractions from the DEAE-Sephadex column that were pooled was of the same specific activity. (ii) One protein band was evident after polyacrylamide gel electrophoresis at pH 8.0 (Fig. 1A). The position of the protein band was identical to the position of the band demonstrating enzymatic activity. (iii) Only one protein band was visible after SDS-polyacrylamide gel electrophoresis of the dissociated enzyme (Fig. 1B).

Molecular weight of the enzyme

The apparent molecular weight of the enzyme was determined in the presence and absence of pyruvate and L-aspartic semialdehyde, both in the

TABLE I

PURIFICATION OF DIHYDRODIPICOLINATE SYNTHASE

Procedure	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units per mg protein)	Per cent yield	Purification
Initial extract	260	9360	6500	1.44	100	1
Heat, 70°C for 10 min	232	9570	1450	6.6	102	4.6
35% Ethanol	550	8792	482	18.2	94	12.6
Streptomycin sulfate	440	8800	440	20	94	13.8
Ammonium sulfate fractionation	41.5	8652	114	74	92	51.4
Sephadex G-200	58.5	6435	14.3	450	69	312
DEAE-Sephadex	56	3050	2.76	1103	33	766

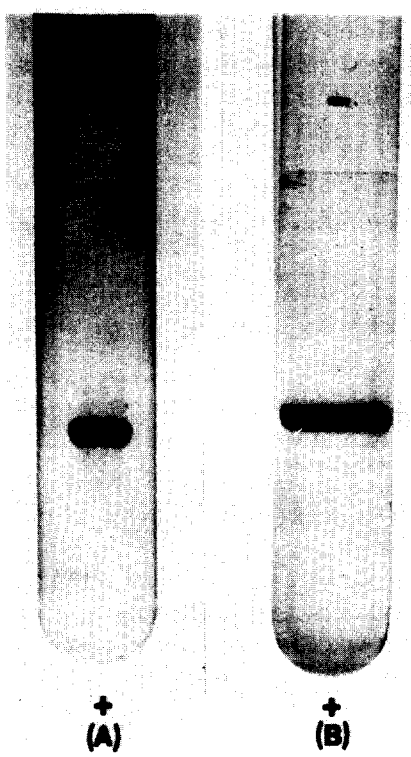

Fig. 1. Polyacrylamide gel electrophoresis of dihydrodipicolinic acid synthase. (A) protein band after polyacrylamide gel electrophoresis of 80 μg of purified enzyme. (B) Protein band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 40 μg of purified enzyme, previously dissociated in mercaptoethanol and sodium dodecyl sulfate. The direction of protein migration was from top to bottom.

presence of high and low concentrations of NaCl using calibrated Sephadex G-200 or Biogel P-200 columns. There were only minor differences in the values obtained for the apparent molecular weight when the enzyme was eluted with buffer containing 0.1 M NaCl or 1 M NaCl either alone or with pyruvate (0.05 M) or L-aspartic semialdehyde (0.01 M) added. The largest apparent molecular weight determined for the enzyme, 117 500 ± 500, was obtained in the presence of 0.05 M Tris · HCl (pH 8.0), 0.01 M NaCl. The smallest apparent molecular weight, 108 000 ± 500, was found when the elution buffer (0.05 M Tris · HCl, pH 8.0) contained 1 M NaCl and 0.05 M pyruvate. As there was no major change in the apparent molecular weight in the presence of NaCl, pyruvate, and L-aspartic semialdehyde, these substances do not cause association or dissociation of subunits of the enzyme.

Subunit molecular weight

The subunit molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 28 000. Electrophoresis of the dissociated enzyme yielded only on band (Fig. 1B). These data are consistent with the enzyme having four subunits of identical size.

Enzyme kinetics

The Micahelis constants of the purified enzyme were determined in the conventional manner with the use of Lineweaver-Burk plots. The K_m (L-aspartic semialdehyde) was approximately 5.0 mM at fixed concentrations of pyruvate between 5 mM and 20 mM. The K_m (pyruvate) was 2.5 to 3.0 mM at fixed L-aspartic semialdehyde concentrations between 6.58 mM and 13.16 mM. The Lineweaver-Burk plots were linear in all cases, when care was taken to measure initial velocities. This precaution was especially crucial in measurement of the K_m (pyruvate) value, for reasons explained in a later section.

Effect of chemical denaturants on the enzyme

The inactivation of the enzyme by urea, ethanol, sodium dodecyl sulfate, and guanidine · HCl was determined as described in Table II. Dihydrodipicolinate synthase was incubated with each denaturant in the presence of either a high (2 M) or a low (0.01 M) NaCl concentration and in the presence and absence of each substrate. After incubation in several concentrations of each denaturant, the enzyme was assayed in normal reaction mixture. These data were used to calculate $C_{0.5}$ values. The $C_{0.5}$ value is defined as the concentration of each denaturant producing 50% inactivation. Pyruvate protected significantly against inactivation by all of the chemical denaturants. Sodium chloride (2 M) protected against inactivation by sodium dodecyl sulfate, guanidine · HCl, and urea, but somewhat facilitated inactivation by ethanol. L-Aspartic semialdehyde had no significant effect on inactivation by sodium dodecyl sulfate and ethanol; it rendered the enzyme slightly more sensitive to inactivation by guanidine · HCl and urea.

Thermodynamics of thermal inactivation of dihydrodipicolinate synthase in the abscence of substrates

Before studying the effect of NaCl and substrates on inactivation of dihydro-

TABLE II

$C_{0.5}$ VALUES FOR DIHYDRODIPICOLINATE SYNTHASE TREATED WITH CHEMICAL DENATURANTS

The $C_{0.5}$ values are defined as the concentration of each denaturant producing 50% inactivation. $C_{0.5}$ values were determined by incubating the enzyme (specific activity, 1100 units per mg of protein) in the presence of varying concentrations of denaturant and then assaying 0.05 ml of this solution in 0.5 ml (final volume) of reaction mixture. The samples all contained 0.05 M sodium barbital buffer (pH 8.0) and either 2 M or 0.01 M NaCl. Pyruvate and L-aspartic semialdehyde (L-ASA) concentrations were 0.05 M and 0.01 M, respectively. The concentration of denaturant causing 50% inactivation was determined from graphs relating the percent of original activity remaining as a function of concentration of denaturant. The activity of the enzyme in the presence of NaCl, pyruvate, or L-aspartic semialdehyde without the addition of denaturant was considered to be 100%. In those cases in which the highest concentration of denaturant was not sufficient to cause 50% inactivation or allow accurate extrapolation to 50% inactivation, the $C_{0.5}$ value is given as being greater than (>) the highest concentration of denaturant used. The enzyme was incubated in denaturant for 60 min at room temperature, when ethanol, urea, and guanidine·HCl were the denaturants. When sodium dodecyl sulfate was used, incubation was for 30 min at 37°C.

Denaturant	Substance added	$C_{0.5}$ values	
		2 M NaCl	0.01 M NaCl
Dodeayl sulfate	None	0.021%	0.02%
	Pyruvate	>0.03%	0.4%
	L-ASA	0.023%	0.02%
Guanidine — HCl	None	1.5 M	1.2 M
	Pyruvate	>1.5 M	>1.5 M
	L-ASA	1.38 M	0.8 M
Urea	None	>5 M	4.9 M
	Pyruvate	>5 M	>5.0 M
	L-ASA	5.1 M	4.5 M
Ethanol	None	32.5%	38.5%
	Pyruvate	>35%	>40%
	L-ASA	29%	38.5%

dipicolinate synthase by another denaturant, heat, a thermodynamic analysis was made of thermal inactivation of the enzyme in the absence of substrates. The rate of thermal inactivation of the enzyme was determined at several temperatures between 74 and 79°C (Fig. 2A). The thermal inactivation rate constant (k) was determined for each temperature. The slope of the line obtained by plotting log k versus the reciprocal of the absolute temperature (Fig. 2B) is equal to $E_a/2.3\,R$. The activation energy (E_a) for thermal inactivation of the enzyme is 117 000 cal/mol. The change in free energy ($\Delta F^{\ddagger}$), change in enthalpy ($\Delta H^{\ddagger}$), and change in entropy ($\Delta S^{\ddagger}$), due to formation of the activated complex in the denaturation process [16,17], were determined to be 23 400 cal/mol, 113 500 cal/mol, and 259 cal/degree/mol, respectively.

Effect of NaCl, pyruvate, and L-aspartic semialdehyde on thermal inactivation

The effect of the substrates, pyruvate and L-aspartic semialdehyde, on thermal inactivation was determined in the presence of low (0.01 M) and high (2 M) concentrations of NaCl. The enzyme was heated for 30 min at various temperatures and then was assayed for enzymatic activity. The activity,

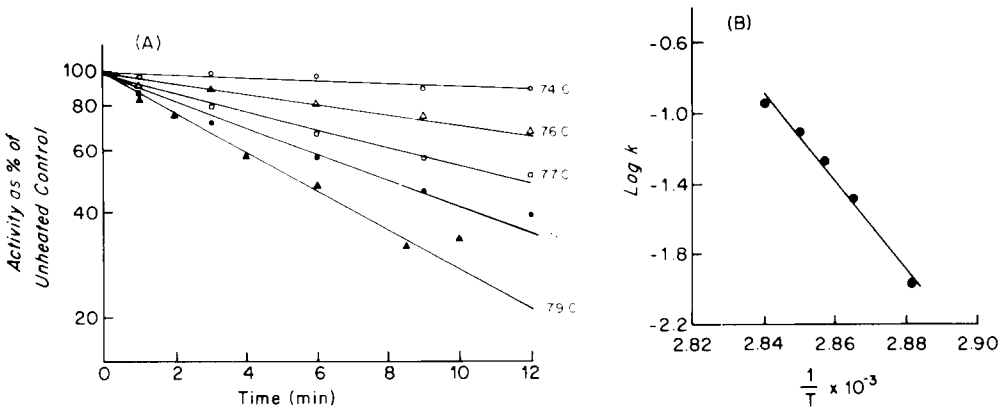

Fig. 2. Kinetics of heat inactivation of dihydrodipicolinic acid synthase and the Arrhenius plot derived from these data. The enzyme was heated in buffer containing 0.05 M sodium barbital (pH 8.6) and 2 M NaCl for various periods of time at the indicated temperatures and then assayed for enzymatic activity. The *k* values derived from the results in (A) were used for the Arrhenius plot (B). The enzyme had a specific activity of 13.2 units per mg protein. The r values for the data obtained at 74, 76, 77, 78, and 79°C (A) are −0.957, −0.996, −0.995, −0.995, and −0.988, respectively. The *r* value for the data presented in (B) is −0.994.

expressed as per cent of the activity of an unheated sample, was plotted as a function of temperature (Fig. 3). The resulting curves probably can be considered melting curves: i.e. the loss in activity probably resulted from heat-induced unfolding of the enzyme molecule. The temperature needed to inactivate half of the enzyme activity is defined as the $t_{0.5}$ value. A high concentration of NaCl or pyruvate enhanced the thermostability of the enzyme. Inactivation by heat in the presence of L-aspartic semialdehyde is more complex (Fig. 3). There is as much as a 50% loss of enzyme activity in the presence of L-aspartic semialdehyde that occurs at relatively low temperatures. At much higher temperatures, the activity disappears. Thus, two $t_{0.5}$ values were calculated when L-aspartic semialdehyde was present. The first and the second $t_{0.5}$ values ($t_{0.5} - 1$ and $t_{0.5} - 2$) represent the temperatures that reduced the activity to 75% and 25% of the original activity (Fig. 3). The $t_{0.5} - 1$ values in 0.01 M and 2 M NaCl (35 and 40°C, respectively) were much lower than the $t_{0.5}$ values for the appropriate control samples (69 and 76°C, respectively). On the other hand, the $t_{0.5} - 2$ values in 0.01 M and 2 M NaCl were 13 and 7°C higher than the $t_{0.5}$ value for the controls. The thermostability in L-aspartic semialdehyde was of approximately the same magnitude as that in pyruvate, if only the $t_{0.5} - 2$ value obtained for the sample heated in L-aspartic semialdehyde is considered. As was the case with stabilization by pyruvate, the $t_{0.5} - 2$ value was independent of the NaCl concentration.

The irreversible inactivation of the enzyme in the presence of L-aspartic semialdehyde or pyruvate is attributed to the instability of the enzyme rather than to the instability of pyruvate or L-aspartic semialdehyde, since the inactivation process is cooperative. Also, when the enzyme was heated in the presence of previously heated L-aspartic semialdehyde and pyruvate, it was protected from complete inactivation at temperatures below 80°C.

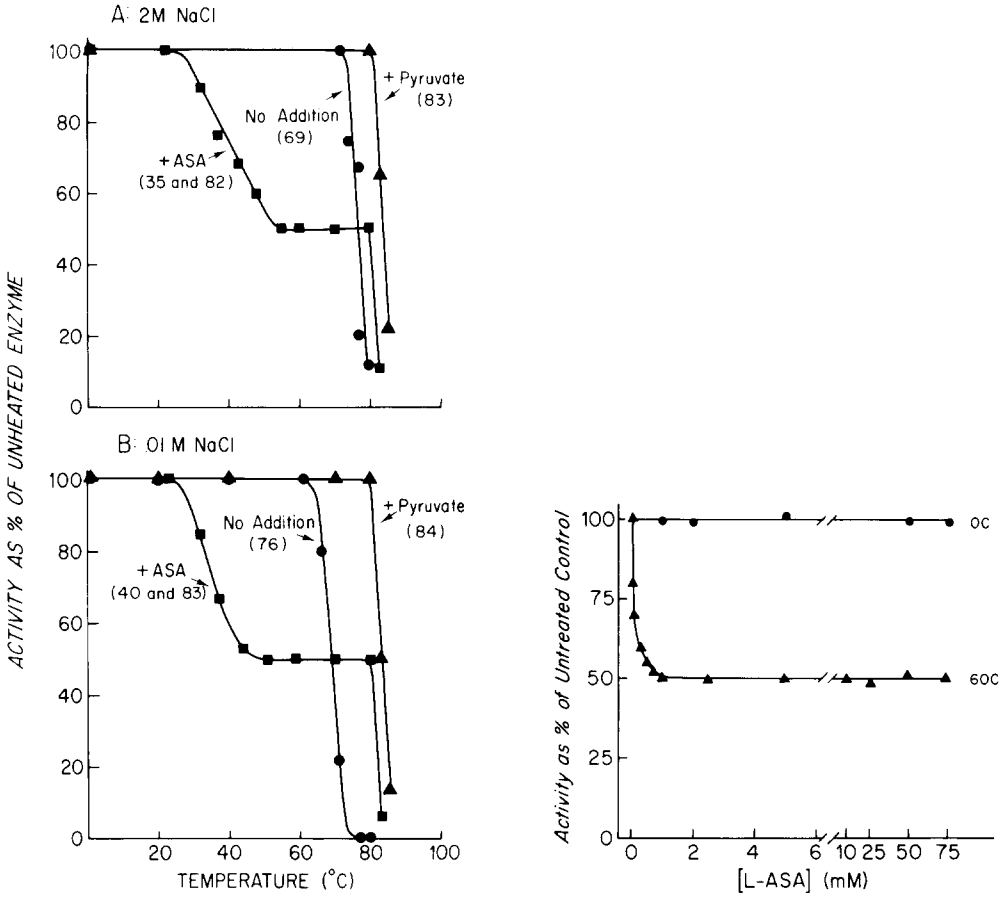

Fig. 3. Effect of the addition of pyruvate and L-aspartic acid semialdehyde (L-ASA) on the activity of the enzyme heated in the presence of relatively high and low concentrations of NaCl. In all cases the buffer consisted of 0.05 M sodium barbital (pH 8.6) and either 2 M NaCl (A) or 0.01 M NaCl (B). When pyruvate or L-ASA were added, the final concentrations were 0.01 M and 0.005 M, respectively. After heating for 30 min the samples were assayed immediately for enzymatic activity. The activity was compared to the activity of the enzyme held at 0°C for 30 min. The numbers in parenthesis represent the $t_{0.5}$ values; the temperatures producing 50% inactivation in 30 min. For the enzyme in the presence of L-aspartic semialdehyde two numbers are given representing the $t_{0.5}$ −1 and $t_{0.5}$ −2 values; temperatures producing 25% and 75% inactivation, respectively, in 30 min (see text). The enzyme used had a specific activity before heating of 6.98 units per mg protein.

Fig. 4. Effect of L-aspartic semialdehyde (L-ASA) concentration on the extent of inactivation of DHDPA synthase at 0 and 60°C. Duplicate portions of the enzyme sample in 0.05 M sodium barbital (pH 8.6) and 2 M NaCl were adjusted to the indicated concentrations of L-aspartic semialdehyde at 0°C. One set of samples was kept at 0°C, and the other set was heated at 60°C. After 30 min, the samples at 60°C were rapidly cooled to 0°C, and both sets of samples were assayed for enzymatic activity. The enzyme used had a specific activity of 6.98 units per mg protein.

Pyruvate binding and thermal stability

As already discussed, pyruvate increase the thermostability of the enzyme. Inactivation rate constants were determined after heating at 80.1°C in 0.05 M sodium barbital (pH 8.6), 2 M NaCl, and pyruvate concentrations ranging from 1 to 50 mM. Sadoff and associates [18] proposed that the first order thermal

inactivation rate constant (k) is related to the concentration of a stabilizing substrate by the equation $k = a$ [substrate]^n, where a is an integration constant and n is the number of substrate molecules bound per active site. The slope of a plot of log k versus log of substrate concentration should be equal to n. Such a plot was made, and a straight line was obtained, the slope of which was 1.03. Thus, thermal stability is provided by binding of pyruvate to one site per subunit; presumably, to the active site.

Relationship between L-aspartic semialdehyde concentration and partial inactivation by heat

A possible explanation for the maximum inactivation of 50% that occurred in the presence of L-aspartic semialdehyde at moderate temperatures (Fig. 3) is that the concentration of L-aspartic semialdehyde used previously (10 mM) was insufficient to promote complete inactivation. Therefore, the enzyme was heated at 60°C for 30 min in the presence of several different concentrations of L-aspartic semialdehyde. The maximal loss of activity was 50% under these conditions, even when the L-aspartic semialdehyde concentration was as high at 75 mM (Fig. 4). The minimal concentration of L-aspartic semialdehyde inducing 50% inactivation was 1.0 mM.

Specificity of L-aspartic semialdehyde as a promotor of the partial inactivation by heat

Dihydrodipicolinate synthase (specific activity, 13.2 units per mg protein) was incubated at 50°C for 15 min in the presence of the following compounds (10 mM): L-aspartic semialdehyde, D-aspartic semialdehyde, aspartic acid, homoserine, asparagine, formaldehyde, and acetaldehyde. The samples were then assayed for enzymatic activity. The purpose of this experiment was to determine how specific L-aspartic semialdehyde was as an effector facilitating the partial heat inactivation. Of the compounds tested, only L- and D-aspartic semialdehyde were found to promote inactivation.

Effect of pyruvate on the partial inactivation caused by heat in the presence of D-aspartic semialdehyde

An obvious question is whether pyruvate has an effect on the partial inactivation by heat that occurs in the presence of L-aspartic semialdehyde. It is not possible to determine the extent of inactivation of the enzyme in the presence of constant concentrations of L-aspartic semialdehyde and pyruvate, however. At the temperatures used for partial inactivation, enzymatic catalysis would occur resulting in the simultaneous disappearance of pyruvate and L-aspartic semialdehyde and in the appearance of dihydrodipicolinic acid. Whereas D-aspartic semialdehyde does promote inactivation of the enzyme, it cannot be condensed with pyruvate by the enzyme to form product [4]. Therefore, the enzyme was heated at 50°C for 15 min in the presence of D-aspartic semialdehyde (13 mM) and several concentrations of pyruvate. After heating, the enzyme samples were assayed. The results (Fig. 5) indicated that the extent of inactivation decreased as the pyruvate concentration was increased. Pyruvate probably also protects against partial inactivation in the presence of L-aspartic

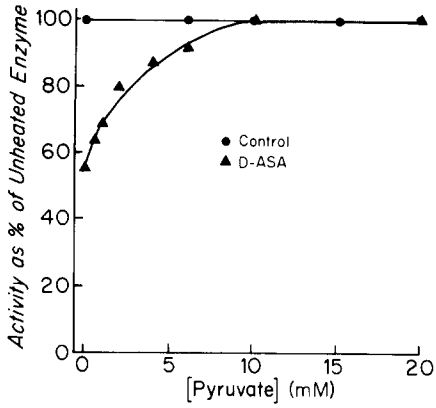

Fig. 5. Relationship of pyruvate concentration to prevention of the heat inactivation facilitated by D-aspartic semialdehyde (D-ASA). No D-aspartic semialdehyde was present in the control. The D-aspartic semialdehyde concentration present in the other samples was 13 mM. All samples contained 0.05 M sodium barbital (pH 8.6) and 2 M NaCl. The samples were heated at 50°C for 25 min and then assayed immediately for enzymatic activity. The activity is expressed as a per cent of the activity possessed by an unheated enzyme sample. The enzyme used had a specific activity of 13.2 units per mg protein.

semialdehyde. Evidence obtained from experiments used for K_m (pyruvate) determinations supports this contention (see below).

Proteolytic modification as a possible explanation of the partial inactivation by heat promoted by L-*aspartic semialdehyde*

One could argue that L-aspartic semialdehyde promoted partial inactivation by changing the conformation of the enzyme, thus, making it more susceptible to proteolytic modification. According to this hypothesis, the proteolytic modification would be the actual cause of the 50% reduction in catalytic efficiency. The heat requirement would be explained as a factor enhancing the rate of activity of the protease. It is possible that the partially purified enzyme preparations used for most of the experiments previously described did contain one or more proteases. However, pure enzyme in the presence of protease inhibitors (1.0 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride) was also inactivated at 50°C in the presence of both L- and D-aspartic semialdehyde. Other evidence ruling out this hypothesis is that the apparent molecular weight of the enzyme does not change after partial inactivation and that the partial inactivation is reversible (see below).

Reactivation following thermal inactivation

At no time was there any recovery of activity of the enzyme observed after thermal inactivation in high or low NaCl concentrations with or without pyruvate. Similarly, there was no recovery of activity after heating at temperatures above 80°C in the presence of L-aspartic semialdehyde. Reactivation of the enzyme partially inactivated at temperatures below 80°C in the presence of L-aspartic semialdehyde was observed, however, when the conditions described below were employed.

Mere removal of L-aspartic semialdehyde from partially inactivated enzyme

was not sufficient to allow reactivation. Dialysis of the inactivated enzyme for five days at 4°C allowed little to no recovery of activity. However, when the dialyzed enzyme was heated at moderate temperatures, increases in activity were observed. The rate of reactivation was found to be directly related to the temperature employed, over the temperature range tested (Fig. 6A). Fig. 6B shows the extent of recovery of activity after heating for 30 min at the temperatures indicated. The results included in Fig. 7 indicate that the rate of reactivation was also increased by pyruvate.

Comparison of the native enzyme with the partially inactivated enzyme

The partially inactivated enzyme was prepared by heating native enzyme,

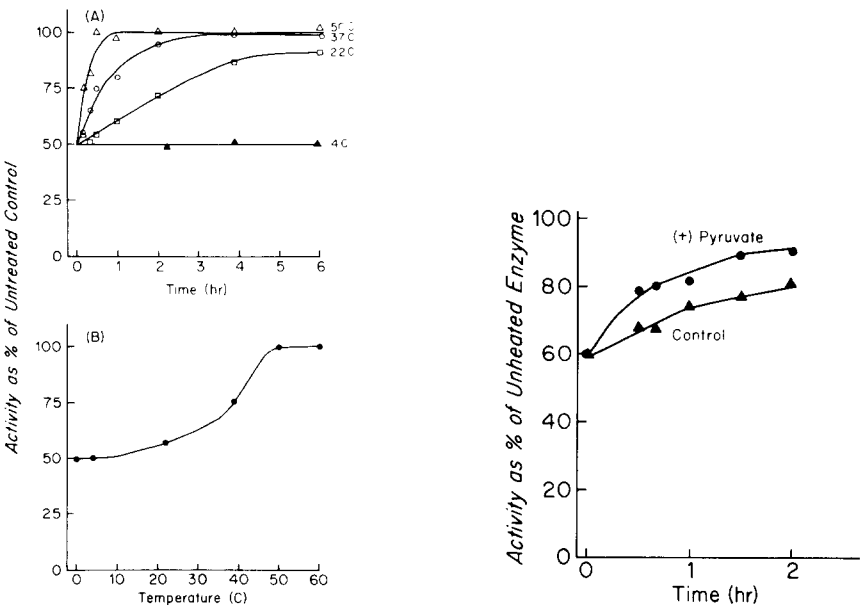

Fig. 6. Effect of temperature on the rate of reactivation of dihydrodipicolinic acid synthase previously inactivated in the presence of L-aspartic semialdehyde. Enzyme was used that had been heated at 50°C for 30 min in buffer containing 0.05 M sodium barbital (pH 8.6), 2 M NaCl, and 0.01 M L-aspartic semialdehyde. After dialysis for 24 h against 2 M NaCl, 0.05 M sodium barbital (pH 8.0) at 4°C, portions of the enzyme sample were placed at the indicated temperatures and assayed at various times thereafter. The top figure (A) presents activity as a function of time of heating. The bottom figure (B) presents the activity as a function of temperature, using the activity obtained after heating for 30 min. The activity is expressed as a per cent of the activity possessed by the enzyme before partial heat inactivation in the presence of L-aspartic semialdehyde. The specific activity of this untreated enzyme was 13.2 units per mg protein.

Fig. 7. Effect of pyruvate on the rate of reactivation of dihydrodipicolinic acid synthase previously partially inactivated in the presence of L-aspartic semialdehyde. Enzyme was used that had been heated at 50°C for 30 min in buffer containing 0.05 M sodium barbital (pH 8.6), 2 M NaCl and 0.01 M L-aspartic semialdehyde. After dialysis for 24 h against 2 M NaCl/0.05 M sodium barbital (pH 8.0) at 4°C, pyruvate (0.05 M) was added to one portion of the sample, the other portion serving as a control. The samples were then assayed after heating at 37°C for various periods of time. The activity is expressed as a per cent of the activity possessed by the enzyme before partial heat inactivation in the presence of L-aspartic semialdehyde. The specific activity of this untreated enzyme was 13.2 units per mg protein.

purified by the heat and streptomycin sulfate steps (specific activity of 13.2 units per mg protein), at 50°C for 30 min in the presence of 10 mM L-aspartic semialdehyde. The L-aspartic semialdehyde was removed by dialysis for 24 h at 4°C against buffer containing 0.05 M sodium barbital and 2 M NaCl (pH 8.0) for the determination of pH optima and enzyme kinetics.

The apparent molecular weight of the native form of dihydrodipicolinate synthase in buffer containing L-aspartic semialdehyde is 111 000 ± 2000 to 114 000 ± 1300, depending on whether the NaCl concentration is 0.1 M or 1 M. The apparent molecular weight of the partially inactivated enzyme was 113 000 at both NaCl concentrations. Therefore, no significant change in molecular weight occurred during partial inactivation.

The pH optima of the native and partially inactivated forms of the enzyme were 8.6. There were no significant differences in the activity profile at pH values above and below 8.6.

As stated previously, the partially denatured form of the enzyme had an activity of approximately 50% that of the native enzyme. It is possible that the conformational change which results in the reduction in activity might be expressed in some other manner such as a change in K_m for pyruvate and/or L-aspartic semialdehyde. Therefore, the K_m for each substrate was determined for both enzyme preparations. The K_m (L-aspartic semialdehyde) was determined using various concentrations of L-aspartic semialdehyde at three approximately saturating concentrations of pyruvate (18, 30, and 50 mM). The K_m (L-aspartic semialdehyde) was nearly independent of pyruvate concentration over the range tested for both enzyme preparations. The K_m (L-aspartic semialdehyde) determined by extrapolation to infinite pyruvate concentration was 5.55 mM for the native and the partially inactivated enzymes. Thus, the partial heat inactivation in the presence of L-aspartic semialdehyde did not result in a significant change in the K_m (L-aspartic semialdehyde).

Determination of the K_m (pyruvate) was more difficult, since the presence of L-aspartic semialdehyde permits heat inactivation at the temperature used for assay (37°C). This was only a problem at low pyruvate concentrations with the native enzyme. At high pyruvate concentrations this inactivation did not occur (Fig. 5). This problem was not experienced with the previously inactivated enzyme, since no further inactivation occurred during assay. Because of the difficulty with the native enzyme, it was very important to measure initial velocities. Further reference to this problem is made in the Discussion. At 9 mM L-aspartic semialdehyde, the K_m (pyruvate) values of the partially inactivated enzyme and native enzyme were 0.91 mM and 1.83 mM, respectively. While these values are not identical, the difference is relatively small and probably not significant. Thus, of the kinetic parameters measured, partial inactivation significantly affected only the catalytic efficiency (V) of the enzyme.

Discussion

Pyruvate and 2 M NaCl were found to enhance stability of dhihydrodipicolinate synthase to heat, and several chemical denaturants. The thermal

stability due to pyruvate was the result of the binding of one pyruvate molecule per subunit, presumably, to the active site. Pyruvate and NaCl probably exert a protective effect against inactivation by shifting the thermodynamic equilibrium between native and denatured states in favor of the native form as a result of binding preferentially to the native form. Pyruvate binds to dihydrodipicolinate synthase of *Escherichia coli* by forming a Schiff's base with an ϵ-amino group of a lysine residue at the active site [19]. Similar binding probably also occurs to the enzyme of *B. licheniformis*. Since a bond of this type has a relatively large dissociation energy, the very significant stabilization provided by pyruvate is reasonable.

The melting curves obtained for the enzyme in the presence of L-aspartic semialdehyde were biphasic. The activity was reduced approximately 50% by heating for 30 min at temperatures between 50°C and 80°C. Only by heating at temperatures above 80°C did the inactivation become complete. The partial inactivation of the enzyme to a form having 50% of the original activity occurred only in the presence of L-aspartic semialdehyde and its stereoisomer, D-aspartic semialdehyde. The aldehyde group of aspartic semialdehyde is apparently required, since asparagine, homoserine, and aspartate had no effect on the enzyme. The possibility that any aldehyde would react with the enzyme causing a partial loss of enzyme activity was ruled out, because neither acetaldehyde nor formaldehyde facilitated heat inactivation. A very low concentration of L-aspartic semialdehyde (1 mM) was required for the partial heat inactivation. Increasing the concentration from 1 to 75 mM had no additional effect. These observations all support the conclusion that the interaction between aspartic semialdehyde and dihydrodipicolinate synthase that results in partial inactivation by heat is very specific.

We do not yet understand the mechanism of the partial inactivation by heat that occurs in the presence of aspartic semialdehyde. There are at least two plausible hypotheses that can be ruled out, however. One of these is that proteolytic modification of the enzyme causes the partial inactivation, the enzyme being more susceptible to protease action in the presence of aspartic semialdehyde. The involvement of heat would be to increase the rate of reaction of the protease. Evidence against this hypothesis is that: (i) purified enzyme in the presence of protease inhibitors is inactivated as readily as partially purified enzyme in the absence of inhibitors; (ii) the apparent molecular weight of the enzyme does not change after partial inactivation in the presence of L-aspartic semialdehyde; and (iii) the inactivation is reversible. A second hypothesis is that isozymes of dihydrodipicolinate synthase exist which vary in their sensitivity to heat. However, there is no evidence for the presence of isozymes of dihydrodipicolinate synthase in *B. licheniformis*. Furthermore, if the biphasic melting curve was due to heat-denaturation of two isozymes, it is unlikely that the process would be reversible for only one of the enzymes.

As indicated in Results, the partial inactivation that occurs at 37°C in the presence of high L-aspartic semialdehyde and low pyruvate concentrations made determination of K_m (pyruvate) values difficult. It was essential to carefully measure initial velocities. Previous use of a fixed-time assay resulted in sigmoid dihydrodipicolinate synthase-pyruvate saturation curves [4]. The report of sigmoid dihydrodipicolinate synthase-pyruvate saturation curves

for the enzyme from *B. subtilis* [3] may also be questionable in the light of these findings.

The specific activity of dihydrodipicolinate synthase in both *B. subtilis* and *B. cereus* increases late in sporulation [3,7,20—22]. This increase may be important to the sporulating cell in that it may be necessary to provide adequate flow of carbon into the lysine biosynthetic pathway at a time when large amounts of dipicolinic acid and diaminopimelic acid are needed. It could be that the increase is caused by induction or derepression. However, no compounds have been discovered that act as inducers or corepressors of this enzyme in sporeformers (for references see Hoganson and Stahly [7]).

It is possible that the level of dihydrodipicolinate synthase activity within the cell might be regulated by the concentrations of L-aspartic semialdehyde and pyruvate in the intracellular pool. When the L-aspartic semialdehyde concentration is high relative to the pyruvate concentration, partial inactivation of dihydrodipicolinate synthase could occur at the growth temperature. An increase in the pyruvate concentration relative to the L-aspartic semialdehyde concentration would be expected to block this inactivation and, hence, increase the specific activity of the enzyme. The turnover rate of the enzyme might be influenced by L-aspartic semialdehyde promoted partial inactivation, if, for example, the partially inactivated enzyme was more susceptible to protease action than the native enzyme.

Acknowledgments

This investigation was supported by National Science Foundation grant BMS-74-10035.

References

1 Powell, J.F. (1953) Biochem. J. 54, 210—211
2 Aronson, A.I., Henderson, E. and Tincher, A. (1967) Biochem. Biophys. Res. Commun. 26, 454—460
3 Chasin, L.A. and Szulmajster, J. (1969) in Spores IV (Campbell, L.L., ed.), pp. 133—147, American Society for Microbiology, Ann Arbor, Michigan
4 Stahly, D.P. (1969) Biochim. Biophys. Acta 191, 439—451
5 Webster, F.H. and Lechowich, R.V. (1970) J. Bacteriol. 101, 118—126
6 Yamakura, F., Ikeda, Y., Kimura, K. and Sasakawa, T. (1974) J. Biochem. 76, 611—621
7 Hoganson, D.A. and Stahly, D.P. (1975) J. Bacteriol. 124, 1344—1350
8 Vold, B., Szulmajster, J. and Carbone, A. (1975) J. Bacteriol. 121, 970—974
9 Walters, J.R. and Stahly, D.P. (1968) Appl. Microbiol. 16, 1605
10 Gornall, A.G., Bardawill, C.J. and Maxima, M.D. (1949) J. Biol. Chem. 177, 751—766
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Andrews, P. (1964) Biochem. J. 91, 222—233
13 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
14 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
15 Laemmli, U.K. (1970) Nature 227, 680—685
16 Eyring, H. (1935) J. Chem. Physics 3, 107—115
17 Whitaker, J.R. (1972) Principles of Enzymology for the Food Sciences, Marcel Dekker, New York
18 Sadoff, H.L., Bach, J.A. and Kools, J.W. (1965) in Spores III (Campbell, L.L. and Halvorson, H.O., eds.), pp. 97—110, American Society for Microbiology, Ann Arbor, Michigan
19 Shedlarski, J.G. and Gilvarg, C. (1970) J. Biol. Chem. 245, 1362—1373
20 Forman, M. and Aronson, A. (1972) Biochem. J. 126, 503—513
21 Hoganson, D.A. and Stahly, D.P. (1975) J. Bacteriol. 124, 1628—1629
22 Andreoli, A.J., Saranto, J., Baecker, P.A., Suchiro, S., Edgardo, E. and Steiner, A. (1975) in Spores VI (Gerhardt, P., Costilow, R.N. and Sadoff, H.L., eds.), pp. 418—424, American Society for Microbiology, Washington, D.C.