BBA 65975

DIHYDRODIPICOLINIC ACID SYNTHASE OF *BACILLUS LICHENIFORMIS*

DONALD P. STAHLY
Department of Microbiology, University of Iowa, Iowa City, Iowa (U.S.A.)

(Received May 27th, 1969)

SUMMARY

Dihydrodipicolinate synthase from *Bacillus licheniformis* was purified approx. 200-fold from that present in initial cell-free extracts. No cofactors were found to be required for activity.

Sigmoid pyruvate–dihydrodipicolinate synthase saturation curves were obtained suggesting pyruvate site cooperativity. This tentative conclusion was also supported by the sigmoid shape of the curve relating degree of heat protection of the enzyme to pyruvate concentration present during heating. Although only L-aspartic semialdehyde could serve as the C-4 substrate, both D- and L-aspartic semialdehyde were found to inhibit the enzyme primarily by decreasing the ability of pyruvate to saturate the enzyme. No cooperative effect was observed between L-aspartic semialdehyde binding sites, either in the presence or absence of D-aspartic semialdehyde.

Thus far, no metabolic inhibitors of dihydrodipicolinate synthase other than the substrate, aspartic semialdehyde have been found.

INTRODUCTION

The first step in the biosynthesis of diaminopimelic acid and lysine in bacteria involves the direct condensation of pyruvate and aspartic semialdehyde. The enzyme which catalyzes this reaction (alternately referred to as the dihydrodipicolinic acid condensing enzyme or as dihydrodipicolinic acid synthase) was first characterized in *Escherichia coli* by YUGARI AND GILVARG[1,2]. These authors found that the *E. coli* enzyme was subject to end product inhibition by lysine. Lysine inhibition of the dihydrodipicolinate synthase of *E. coli* was recently shown by TRUFFA-BACHI *et al.*[3] to exhibit lysine site cooperative interaction.

Control of dihydrodipicolinic acid synthase in spore-forming microorganisms is complicated by the fact that dihydrodipicolinate is not only a precursor of L-lysine but probably also of dipicolinic acid. Dipicolinic acid is the compound present in bacterial endospores and absent from vegetative cells, which is associated with heat resistance[4–6]. BACH AND GILVARG[7], and more recently CHASIN AND SZULMAJSTER[8], have demonstrated sporulating cell extract catalyzed synthesis of dipicolinic acid from L-aspartic semialdehyde and pyruvate. Auxotrophs of *Bacillus megaterium*

lacking dihydrodipicolinate synthase were unable to form dipicolinic acid while auxotrophs blocked further on in the pathway to lysine were able to sporulate normally when the growth medium was supplemented with diaminopimelate and lysine[9]. Thus it is assumed that dipicolinic acid derives from dihydrodipicolinate by enzymatic dehydrogenation.

The dihydrodipicolinate synthases of both *Bacillus cereus*[6] and *Bacillus subtilis*[8] were not inhibited by lysine. Thus, a continued synthesis of dihydrodipicolinate for dipicolinic acid synthesis is not prevented by lysine. Not only is it lysine insensitive, but also the amount of the enzyme is greatly increased in sporulating *B. subtilis* cells[8].

This report outlines some of the characteristics of dihydrodipicolinate synthase from *Bacillus licheniformis*. Included are results of storage stability experiments, a scheme for partial purification of the enzyme, kinetic analysis and some experiments giving indirect information concerning enzyme structure. Unique findings were that the enzyme exhibited pyruvate site cooperative interaction, and that it was inhibited at low concentrations of pyruvate by aspartic semialdehyde, the second substrate. As was the case with the other Bacillus species, no lysine or diaminopimelate induced inhibition was observed.

METHODS AND MATERIALS

Culturing of the microorganisms

B. licheniformis A-5 was used throughout this study. The medium and conditions employed in culturing the organism were those specified previously[10], with the only modification being that the salts mixture included 0.1 g. $CaCl_2 \cdot 2H_2O$ per l of medium.

The above medium was also used for growth of *E. coli* ATCC 9637 and *E. coli* K_{12}.

Protein determination

Protein concentration was determined by the method of LOWRY *et al.*[11] or by the Biuret assay as described by GORNALL *et al.*[12]. For these and other colorimetric determinations a Zeiss PMQII spectrophotometer was used.

Synthesis of aspartic β-semialdehyde

DL-Aspartic β-semialdehyde was synthesized by the methods outlined by BLACK AND WRIGHT[13]. These procedures, some of which were modified slightly as described below, were also employed for the preparation of the D and L isomers of aspartic semialdehyde. Deacylation of the *N*-acetyl L-allylglycine in the *N*-acetyl DL-allylglycine mixture was accomplished by use of commercial hog kidney acylase I (Sigma Chemical Co.), whereas BLACK AND WRIGHT[13] had their own enzyme prepared in the laboratory. We found that a higher concentration (100 mg/l) of the commercial enzyme was required to affect deacylation in a reasonable period of time than that specified by BLACK AND WRIGHT[13]; *i.e.* 5.64 mg/l. The extent of deacylation was followed polarimetrically rather than by manometric ninhydrin–CO_2 measurement.

Homoserine dehydrogenase in initial cell-free extracts of *E. coli* K_{12} was used for measurement of the quantity of the L-aspartic semialdehyde in the L- and DL-aspartic semialdehyde preparations. The quantity of D-aspartic semialdehyde prepared was determined by ninhydrin assay[14].

Assay of dihydrodipicolinic acid synthase

The assay employed for measurement of dihydrodipicolinic acid was a modification of the *o*-aminobenzaldehyde assay developed by YUGARI AND GILVARG[1]. As the developers indicated, the advantage of this assay over others is its specificity and lack of interfering side reactions. They pointed out, however, that the rate of reaction of *o*-aminobenzaldehyde with dihydrodipicolinate is rather slow and hence, there is a long lag before the reaction shows uniform increases in color with time. Therefore, the assay was modified to avoid this difficulty and also to increase the sensitivity of the assay. Instead of measuring the formation of the product kinetically during the reaction, portions of the reaction mixture were periodically removed to 0.22 M citrate–0.55 M Na_2HPO_4 buffer, pH 5.0. This procedure stops the reaction and also increases the optical absorption of the dihydrodipicolinate–*o*-aminobenzaldehyde adduct. At pH 5.0, the optimum absorption occurs at 520 mμ. After stopping the reaction, the color continues to increase for a period of approx. 80 min, probably because of the slow reaction of dihydrodipicolinic acid with *o*-aminobenzaldehyde. Therefore, samples were routinely left at room temperature for 2 h prior to spectrophotometric assay. The color was stable for a period of at least 6 h.

By use of this modified assay, a linear relationship was obtained between velocity and reaction time, and velocity and amount of enzyme.

Unless otherwise specified, the reaction mixture contained: Tris–HCl buffer, pH 8.4 or pH 8.0, 0.1 mmoles; L-aspartic β-semialdehyde, 4.5 μmoles; potassium pyruvate, 25 μmoles; *o*-aminobenzaldehyde, 0.5 mg; and enzyme solution. The total volume was 0.5 ml.

1 unit of enzyme is defined as that amount catalyzing an increase in $A_{520\ m\mu}$/min = 1.0 at 37°. Specific activity is expressed as units of enzyme per mg of protein.

Other enzymatic assays

Homoserine dehydrogenase was assayed in the direction of aspartic semialdehyde to homoserine and aspartic semialdehyde dehydrogenase in the direction of aspartic semialdehyde to aspartyl phosphate by the methods of PATTE *et al.*[15] and COHEN AND PATTE[16], respectively.

Preparation of initial cell-free extract of E. coli ATCC 9637 for assay of dihydrodipicolinate synthase

During late exponential phase growth, the cells were centrifuged, washed once and resuspended prior to breakage in 0.02 M Tris buffer (pH 7.4). The cells were broken as described for the *B. licheniformis* cells, and after centrifugation at 25 000 × *g* for 15 min, the supernatant was dialyzed against the previously mentioned buffer and used for enzymatic assay.

Purification of B. licheniformis dihydrodipicolinic acid synthase

Step 1. The cells were harvested during exponential growth at 230 Klett units No. 54 (as measured by a Klett–Summerson photoelectric colorimeter) by centrifugation at 15 000 × *g* at 4° in an International B-20 refrigerated centrifuge or by use of a Sharples Super-centrifuge. The cells were washed once in buffer, centrifuged again, and then were frozen until ready for use or immediately resuspended in buffer in preparation for cell breakage. As will be discussed subsequently, the buffer which

rendered the enzyme the most stable was 0.1 M Tris (pH 8.0), 2 M NaCl. The cells were broken by passage through a modified Aminco–French pressure cell[17]. After removal of whole cells and cell debris by centrifugation at 25 000 × *g* for 15 min, the supernatant solution was usually dialyzed against the previously mentioned buffer. The extracts were either utilized as such or the enzyme was purified as described below. The cell-free extract and purified fractions were stored at 0°.

Step 2. The initial cell-free extract was adjusted to pH 8.5 with concentrated Tris, placed in a water bath at 70°, and swirled until the temperature of the contents reached 70°. After 10 min at this temperature with occasional swirling, the flask was rapidly cooled in an ice-water bath. The heated suspension was centrifuged at 25 000 × *g* for 15 min to sediment the denatured protein. The pellet was extracted with an additional volume of buffer and recentrifuged. The supernatant was combined with the original supernatant and the pellets were discarded.

Step 3. The combined supernatant was adjusted to 20 mM potassium pyruvate and then heated at 80° for 10 min. The heated suspension was centrifuged at 25000 × *g* for 15 min to sediment the denatured protein. The pellet was extracted with an additional volume of buffer and recentrifuged. The supernatant was combined with the original supernatant and the pellets were discarded.

Step 4. The supernatant from Step 3 was concentrated by precipitation with saturated $(NH_4)_2SO_4$. The precipitate was dissolved in a small volume of 0.1 M Tris, pH 8, 0.5 M NaCl and was dialyzed against the same buffer for 6 h. The enzyme (5 ml) was placed on a column (2.5 cm × 80 cm) of Sephadex G200 at 4° that was previously equilibrated with 0.1 M Tris, pH 8, 0.5 M NaCl. The enzyme was than eluted as a single peak with the same buffer. The most active eluant fractions from the Sephadex column were combined and adjusted to 2 M NaCl.

Step 5. The combined fractions from the Sephadex column were concentrated by ultrafiltration through an Amicon UM-1 diaflo ultrafiltration membrane. The concentrated extract was dialyzed against 0.1 M NaCl, 0.1 M Tris, pH 8.0 for 6 h. After dialysis, 2 ml of the concentrated enzyme was placed at 4° on a DEAE-cellulose column (25 cm × 2.5 cm) previously equilibrated with 0.1 M NaCl, 0.1 M Tris, pH 8.0. The enzyme was eluted with a linear gradient of 0.1–0.5 M NaCl in 0.1 M Tris, pH 8.0. The

TABLE I

PURIFICATION OF *B. licheniformis* DIHYDRODIPICOLINATE SYNTHASE

Procedure	*Vol. (ml)*	*Total activity (units)**	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Yield (%)*	*Purification*
Initial extract	34.5	1098	2015	0.544	100	1
Heat at 70° for 10 min	40.3	1050	315	3.34	95.6	6.1
Heat at 80° for 10 min in presence of 20 mM pyruvate	42.3	903	240	3.77	82	6.9
Sephadex G200	8.15	767	31.2	24.6	70	45.2
DEAE-cellulose	144	248	2.4	103	23	190

* 1 unit: $\Delta A_{520\ m\mu}$ per min = 1.0 at 37°.

buffer–NaCl reservoirs each held 500 ml. The enzyme activity was eluted as a single peak. The active fractions were combined and adjusted to 2 M NaCl as soon as possible because of the instability of the enzyme at low ionic strength.

A summary of a typical purification is shown in Table I.

RESULTS

Optimum pH for dihydrodipicolinate synthase activity

Dihydrodipicolinate synthase from *B. licheniformis* exhibited maximum activity at pH 8.4 in initial cell-free extracts, but after the heat steps employed in purification, the optimum shifted to pH 8.0.

Stability of dihydrodipicolinate synthase during storage

The enzyme was found to require high ionic strength for storage stability at 0° at all stages of purification. The heated enzyme preparation lost activity somewhat slower than the initial cell-free extract. Fig. 1 illustrates the stabilizing effect of NaCl on initial cell-free extract. At a protein concentration of 9.4 mg/ml, 2 M NaCl was required to maintain stability. The amount of NaCl required for stability is inversely related to the protein concentration; *e.g.* at 37.2 mg/ml protein concentration, 1 M NaCl rendered the enzyme stable at 0° for at least 2 weeks.

The optimum pH for storage stability in 2 M NaCl at 0° was determined to be

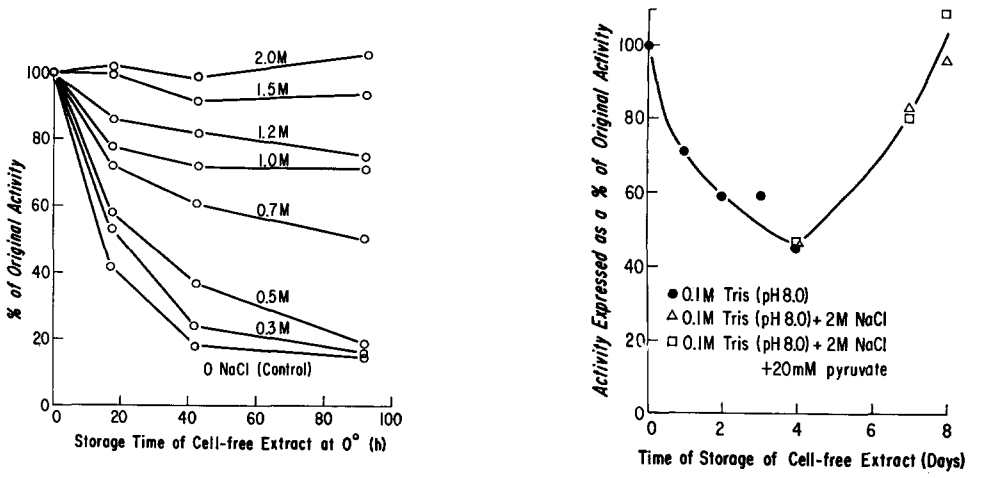


Fig. 1. The effect of NaCl concentration on storage stability of dihydrodipicolinate synthase. The initial cell-free extract (9.4 mg/ml) was dialyzed 8 h against 0.01 M Tris buffer, pH 8.0. The specific activity at this time (0 time on the figure) was 0.457 unit/mg protein. Separate samples were adjusted to the NaCl concentration indicated with 4 M NaCl. The samples were stored at 0° and were assayed at periodic intervals thereafter. DL-Aspartic semialdehyde was employed in the reaction mixtures.

Fig. 2. Reversible inactivation and reactivation of dihydrodipicolinate synthase. Cells were broken in 0.1 M Tris buffer, pH 8.0. The initial cell-free extract was heated at 70° for 10 min, was centrifuged to remove precipitated protein, and then was dialyzed against 0.1 M Tris, pH 8.0. The specific activity of the enzyme at the beginning of dialysis (0 h in the figure) was 0.72 unit/mg protein. DL-Aspartic semialdehyde was employed in the reaction mixture. Other details are presented in the text.

pH 8.0, using potassium phosphate and Tris buffers between pH 6.5 and 9.0.

The loss of activity in the presence of low ionic strength is reversible as is shown in Fig. 2. When the cell-free extract was kept in 0.1 M Tris buffer (pH 8.0) for 4 days at 4°, only 45% of the original activity remained. Continued dialysis of this enzyme at 4° against 0.1 M Tris buffer (pH 8.0), 2 M NaCl or the same buffer *plus* 20 mM pyruvate allowed a slow but complete recovery of activity. The process of dialysis is not essential for reactivating the enzyme. Recovery of activity also occurred when 4 M NaCl was added directly to the inactivated enzyme to adjust to a final concentration of 2 M NaCl.

The NaCl requirement for storage stability is not specific. Similarly high concentrations of LiCl, KCl, Tris–HCl, and potassium phosphate buffer also stabilized the enzyme to inactivation during storage. Relatively lower concentrations of $CaCl_2$ (10 mM), $MnCl_2$ (1 and 10 mM), $MgCl_2$ (1 and 100 mM), and $ZnCl_2$ (10 mM) failed to provide enzyme stability. Pyruvate (20 mM) which stabilizes the enzyme to heat inactivation, failed to stabilize the enzyme during storage. Mercaptoethanol at 1.0 and 5.0 mM concentrations was also without beneficial effect.

Repeated freezing and thawing resulted in loss of enzyme activity.

Enzyme kinetics

The saturation curve for the dihydrodipicolinate synthase–pyruvate complex was determined at three different aspartic semialdehyde concentrations, and the results are presented in Fig. 3. Sigmoid kinetics indicative of pyruvate site interactions were readily apparent, the extent of cooperativity being increased as the aspartic semialdehyde concentration was raised. Similar results have been obtained using initial cell-free extracts. It appears that aspartic semialdehyde exerts its inhibitory effect on the ability of pyruvate to saturate the enzyme and not on the maximal velocity. Table II indicates the effect of aspartic semialdehyde concentration on the pyruvate concentration required for half maximal velocity ($S_{1/2}$).

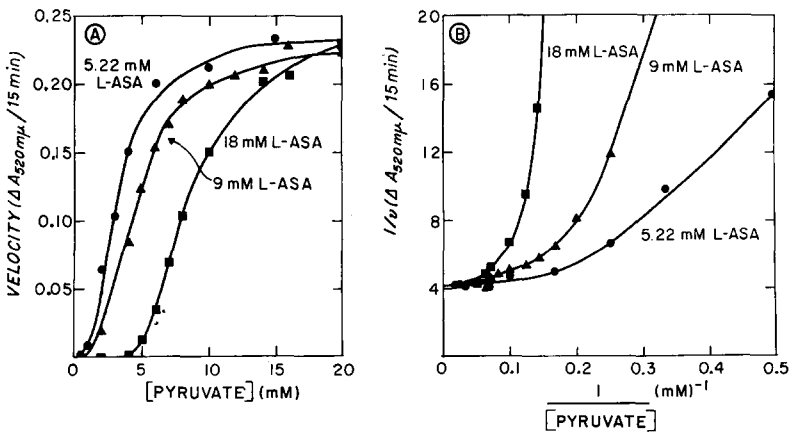


Fig. 3. Saturation curves (A) and double reciprocal plots (B) for the dihydrodipicolinate–pyruvate complex at 3 different L-aspartic semialdehyde (L-ASA) concentrations. The enzyme utilized was purified through the DEAE-cellulose step and had a specific activity of 112 units/mg protein. The quantity of protein used in each reaction mixture was 0.567 μg.

TABLE II

EFFECT OF L-ASPARTIC SEMIALDEHYDE CONCENTRATION ON $[S_{1/2}]$ (PYRUVATE CONCENTRATION GIVING $^1/_2\, v_{max}$)

For details concerning experimental procedure, see Fig. 3.

L-Aspartic semialdehyde concn. (mM)	*($S_{1/2}$) (mM)*
5.22	3.75
9.0	5.5
18.0	9.3

The presence of other enzymes in the "purified" dihydrodipicolinate synthase might possibly explain the apparent allosteric kinetics. No homoserine dehydrogenase or aspartic semialdehyde dehydrogenase activity was demonstrable in the purified enzyme preparation. Although the presence of other pyruvate utilizing enzymes has not been ruled out, this possibility has been minimized by the inclusion in the purification procedure of a 70° heat step in the absence of pyruvate. The succeeding 80° heat step involves inclusion of pyruvate which might stabilize other pyruvate-utilizing enzymes as well as the dihydrodipicolinate synthase. Further evidence that the sigmoid pyruvate–dihydrodipicolinate synthase saturation curve obtained is not an artifact, lies in the fact that Michaelis–Menten kinetics were observed when similar data was obtained using dihydrodipicolinate synthase in *E. coli* ATCC 9637 initial cell-free extract. This extract no doubt contained several enzymes other than dihydrodipicolinate synthase for which pyruvate and aspartic semialdehyde serve as substrates. This observation with the *E. coli* enzyme is in agreement with that of YUGARI AND GILVARG[1], who employed the same strain of *E. coli*.

Pyruvate site cooperativity with the *B. licheniformis* enzyme can be demonstrated in the absence of aspartic semialdehyde. Fig. 4 shows the results of an experiment which involved heating of initial cell-free extracts at 80° for 15 min in the presence of various concentrations of pyruvate prior to assay.

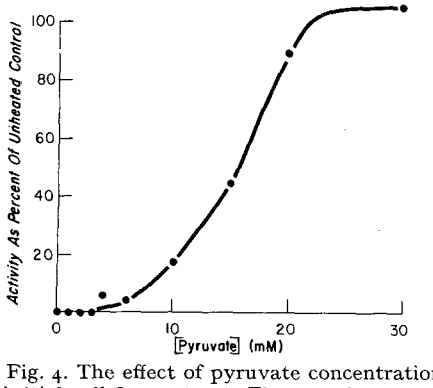
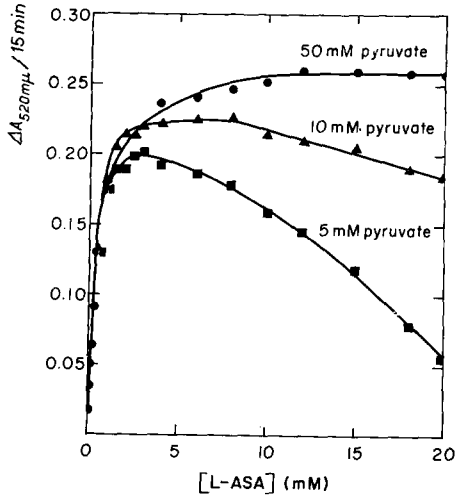


Fig. 4. The effect of pyruvate concentration on heat stability of dihydrodipicolinate synthase in initial cell-free extract. The protein concentration during heating was 25.8 mg/ml and the pH of the cell-free extract was 8.5. The specific activity of the unheated control was 0.66 units/mg protein. 0.05 ml of each enzyme preparation was assayed by the normal procedure.



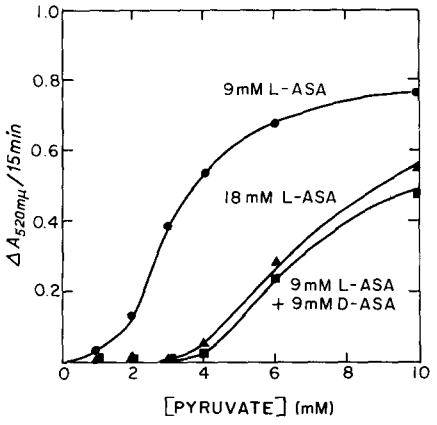


Fig. 5. Saturation curve for the dihydrodipicolinate–L-aspartic semialdehyde (L-ASA) complex at 3 different pyruvate concentrations. The enzyme utilized was purified through the DEAE-cellulose step and had a specific activity of 112 units/mg protein. The quantity of protein used in each reaction mixture was 0.567 μg.

Fig. 6. The effect of the D and L isomers of aspartic semialdehyde (ASA) on the dihydrodipicolinate synthase–pyruvate saturation curve. The enzyme used was purified through the 2 heat steps and had a specific activity of 4.18 units/mg protein. The amount of protein used in each reaction mixture was 0.0515 mg.

Fig. 5 presents the saturation curves for the dihydrodipicolinate synthase L-aspartic semialdehyde complex obtained at three pyruvate concentrations. The inhibitory effect of high aspartic semialdehyde concentration was again obvious. Pyruvate was shown to reverse aspartic semialdehyde inhibition. No detectable inhibition occurred in the presence of 50 mM pyruvate. This finding is consistent with the data of Fig. 3.

No substrate site interaction was observed with aspartic semialdehyde. The Hill number[18] derived from the data of Fig. 5 with 50 mM pyruvate was 1.075. The K_m (L-aspartic semialdehyde) was 0.765 mM (see Fig. 7B; 0 mM D-aspartic semialdehyde curve).

No enzymatic activity was observed when L-aspartic semialdehyde was replaced by D-aspartic semialdehyde. D-Aspartic semialdehyde did have an effect on the enzyme, however, as is demonstrated by the results shown in Fig. 6. D-Aspartic semialdehyde increased the $S_{1/2}$ (pyruvate). The sigmoidicity of the pyruvate–dihydrodipicolinate synthase saturation curve was approximately the same when 9 mM D-aspartic semialdehyde was used in conjunction with 9 mM L-aspartic semialdehyde as when 18 mM L-aspartic semialdehyde was employed alone. D-aspartic semialdehyde inhibition of activity was reversable by high concentrations of pyruvate as was the case with L-aspartic semialdehyde.

In addition to increasing the [$S_{1/2}$] ([pyruvate]), D-aspartic semialdehyde also was found to increase the K_m (L-aspartic semialdehyde) and slightly decrease the v_{max} (Fig. 7). No L-aspartic semialdehyde site cooperativity was observed in the presence or absence of D-aspartic semialdehyde.

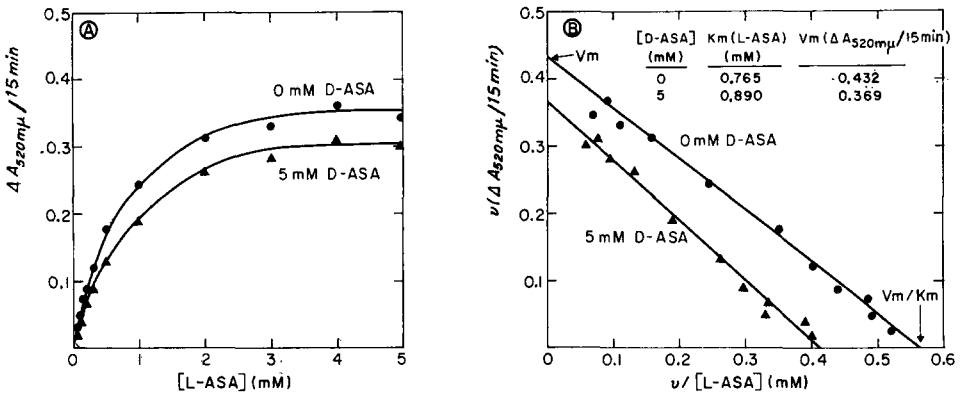


Fig. 7. Effect of D-aspartic semialdehyde (D-ASA) on the dihydrodipicolinate synthase–L-aspartic semialdehyde (L-ASA) saturation curve. (A) Normal plot of velocity *versus* substrate concentration at 0 and 5 mM D-aspartic semialdehyde. (B) Hofstee plot of the same data. The enzyme preparation used was purified through the Sephadex step and possessed a specific activity of 23 units/mg protein. 0.005 mg of protein was used in each assay.

Effect of urea on enzyme activity

The effect of urea concentration on enzyme activity is shown in Fig. 8. Exposure to urea at a concentration of 6 M or higher resulted in complete inactivation of the enzyme. When inactivated enzyme was dialyzed against 2 M NaCl, 0.1 M Tris (pH 8.0) for 4 h, 89% reactivation occurred. Dialysis for an additional 4 h resulted in complete reactivation. No reactivation occurred in 0.1 M Tris in the presence or absence of

Fig. 8. Effect of urea concentration on activity of dihydrodipicolinate synthase. Initial cell-free extract was adjusted to various concentrations of urea and the activity was determined on 0.05 ml of each sample after incubation at room temperature for 2 h. The protein concentration of each tube during inactivation was 12.85 mg/ml. The specific activity of the untreated enzyme was 0.775.

Fig. 9. Effect of protein concentration on rate of reactivation of urea-inactivated dihydrodipicolinate synthase. An enzyme preparation purified through the 80° heat step was incubated in 7 M urea for 2 h at 23°. It was diluted as indicated in the figure with reactivation buffer (2 M NaCl, 0.1 M Tris (pH 8), 50 mM pyruvate) at 0 min. The final urea concentration in the 3 samples was 0.2 M. The temperature during reactivation was 23°. An equal amount of protein was used for assay of each of the 4 samples. The "untreated control" consisted of the enzyme unexposed to urea and was included to give an indication of stability of the reaction mixture which was prepared for the entire experiment prior to 0 min.

pyruvate (50 mM). Reactivation in the high ionic strength buffer proved to be dependent both on enzyme concentration and on temperature (Figs. 9 and 10). The relationship observed between enzyme concentration and rate of reactivation would be expected if reactivation depended on polymer formation from subunits. In these experiments reactivation occurred in the presence of pyruvate. Reactivation was not dependent on pyruvate, but the rate was accelerated in its presence. Reactivation appeared to be a higher than first order reaction (see especially Fig. 10), thus further suggesting that an active structure may require the association of subunits.

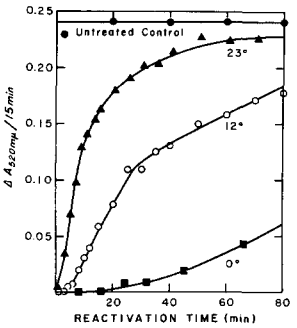


Fig. 10. Effect of temperature on rate of reactivation of urea-inactivated dihydrodipicolinate synthase. An enzyme preparation different from that used in the experiment illustrated in Fig. 9 but similarly purified was inactivated in 7 M urea for 2 h at 23°. At 0 min, it was diluted in 2 M NaCl, 0.1 M Tris (pH 8.0), 50 mM pyruvate to a final protein concentration equivalent to the 1/100 dilution of Fig. 9 and a urea concentration of 0.2 M. The reactivation temperatures were as indicated above. 0.05 ml of each of the 4 samples was assayed in the usual manner. The purpose of the "untreated control" was as indicated in the legend to Fig. 9.

Search for inhibitors or activators of dihydrodipicolinate synthase

Since dihydrodipicolinate synthase is the first enzyme in the biosynthetic branch leading to diaminopimelic acid and lysine, and since the enzyme was demonstrated to have allosteric kinetics, it seemed logical to look for possible inhibitors or activators. The effect of various substances on enzyme activity was determined using (a) a saturating concentration of pyruvate and an unsaturating concentration of aspartic semialdehyde; (b) saturated aspartic semialdehyde and unsaturated pyruvate; and (c) unsaturated pyruvate and aspartic semialdehyde. Thus far, no inhibitors or activators have been found other than the substrate, aspartic semialdehyde. All substances were tested at a final concentration of 10 mM. Aspartate and the amino acids derived from aspartate were tested. These included diaminopimelic acid, lysine, homoserine, methionine, threonine, and isoleucine. Dipicolinic acid was also tested. The adenylates, ATP, ADP, and AMP were without effect. The substance from which aspartic semialdehyde was synthesized, allylglycine, had no effect on activity.

DISCUSSION

Dihydrodipicolinate synthase activity in cell-free extracts from late exponential phase *B. licheniformis* cells seems to reside in a single protein, since only one peak of

activity was recovered subsequent to Sephadex G200 and DEAE-cellulose column chromatography.

The enzyme required high ionic strength for storage stability, and although this requirement was non-specific with regard to ionic species, 2 M NaCl was routinely employed. High ionic strength in the case of other enzymes has been shown to exert associative or dissociative effects. The dissociation of hemoglobin molecules of molecular weight 68 000 into subunits is favored by an increase in ionic strength, probably by reducing the electrostatic interaction[19]. In the case of insulin, an increase in ionic strength results in aggregation[19]. This effect could result if cohesive tendencies due to hydrophobic interaction were antagonized by repulsive electrostatic forces. Addition of a high concentration of a neutral salt would mask the repulsive forces and hence favor aggregation. Precisely what effect high NaCl concentration has on dihydrodipicolinate synthetase is yet to be determined. Both high NaCl and pyruvate may favor an aggregated form, since it has been found that both substances antagonize the inactivating effects of urea and guanidine–HCl, well known dissociative agents (unpublished data).

It has been found that urea-inactivated enzyme is reactivated in the presence of 2 M NaCl, 0.1 M Tris buffer (pH 8), but not in 0.1 M Tris buffer (pH 8). Furthermore, the rate of reactivation was dependent on protein concentration and on temperature. This could be interpreted as indirect evidence for the existence of subunits. At high protein concentration or at high temperature, collision of enzyme subunits would occur frequently, thus allowing aggregation to occur more rapidly than at low protein concentration or low temperature. Also, the fact that the reactivation rate is higher than first order suggests that subunit aggregation is involved.

Although the above evidence suggests that high NaCl does indeed favor subunit aggregation, more direct evidence must be obtained before any definite conclusions can be made. It is possible that aggregation of urea-inactivated enzyme occurs in the low ionic strength buffer even though no activity increase is observed. Exposure of this enzyme to a high NaCl concentration might simply induce a conformational change in the aggregated enzyme, this change being necessary for catalysis to occur. Also the observation that high NaCl antagonizes the inactivating effects of urea and guanidine–HCl might be explained if the conformational state of the enzyme polymer in the presence of high NaCl concentration is more resistant to dissociation than the polymer in a low NaCl concentration environment.

The dihydrodipicolinate synthase of *B. licheniformis* is similar to that of *E. coli*[1] in that no cofactor as yet has been discovered. Unlike the *E. coli* enzyme[2], the *B. licheniformis* dihydrodipicolinate synthase was not inhibited by lysine. It was also found that diaminopimelic acid and dipicolinate exerted no effect on activity. The lack of lysine or diaminopimelate inhibition of activity should be of physiological benefit to sporeformers, since dihydrodipicolinate is required for the synthesis of dipicolinic acid as well as lysine.

Another difference between *B. licheniformis* enzyme and that of *E. coli*, is that the former enzyme exhibits pyruvate site cooperativity while normal Michaelis–Menten kinetics occur with the latter enzyme. Pyruvate site cooperativity was also reported to occur with the partially purified dihydrodipicolinate synthase of *B. subtilis*[20]. The *B. licheniformis* enzyme was subject to inhibition by the substrate aspartic β-semialdehyde. One might conclude that pyruvate site cooperativity observed in the case

of the experiment involving determination of activity at various pyruvate concentrations (Fig. 3) could have been simply due to the obligate presence of the inhibitor, aspartic semialdehyde, in the reaction mixture. It was found, however, that pyruvate site cooperativity could be demonstrated in the absence of aspartic semialdehyde by measuring the degree of heat protection in the presence of variable pyruvate concentration (Fig. 4). Knowledge of how pyruvate site cooperativity and aspartic semialdehyde inhibition of dihydrodipicolinate synthase may be of benefit to *B. licheniformis* must await further experimentation.

The inhibition of aspartic semialdehyde is not on catalytic ability of the enzyme (v_{max}) but on the ability of pyruvate to saturate the enzyme ($[S_{1/2}]$).

One model that has been proposed to explain sigmoid saturation curves, the model according to MONOD *et al.*[21], is based on the assumption that each identical subunit of a multi-subunit enzyme contains a single binding site for substrate. Further, the polymer exists in at least 2 conformational states, the transition from one state to another involving simultaneous changes in all the identical subunits. If a substrate molecule has a greater affinity for one conformational state than for another, then when substrate is added at low concentration, single substrate molecules will preferentially bind to one subunit of the enzyme in the conformational state which has the greatest affinity for it. This will cause a shift of the equilibrium in favor of the enzyme conformation having the greater affinity for the substrate, and therefore, will facilitate the subsequent binding of additional substrate molecules.

Certain other models that attempt to explain sigmoid saturation curves assume the existence of at least 2 substrate binding sites, a catalytic and a regulation site (see ref. 22). It is assumed that binding of the substrate at the regulatory site induces a conformational change in the enzyme which increases the affinity of the catalytic site for the substrate.

From existing data in the literature, it is difficult to determine which of these models is correct. Indeed both may exist in different systems.

The sigmoid pyruvate saturation curves obtained with *B. licheniformis* dihydrodipicolinate synthase could be explained by either the one, or the two-site hypothesis. One could assume that pyruvate binding at the catalytic site at low concentration shifts the equilibrium of the enzyme to a form that binds pyruvate more readily than the other form. Alternatively, pyruvate binding to a regulatory site might be necessary in order to change the enzyme conformation to a form which allows pyruvate binding to the catalytic site.

Aspartic β-semialdehyde could inhibit binding of pyruvate to the enzyme by at least two mechanisms also. It could inhibit if aspartic semialdehyde bound to the catalytic site of a conformational form different from that which binds pyruvate maximally. It could also inhibit by binding to an inhibitor site separate from the catalytic site, the binding in turn causing a conformational change to a form which binds pyruvate poorly. Efforts are currently being made to determine which of these 2 models better describes the situation in the case of *B. licheniformis* dihydrodipicolinate synthase.

Dihydrodipicolinate synthase from *B. licheniformis* is capable of utilizing only the L isomer of aspartic semialdehyde. The D isomer, while not acting as a substrate, did prove to inhibit activity. Although it did inhibit the enzyme by increasing the K_m (L-aspartic semialdehyde) and decreasing the v_{max} slightly (Fig. 7) the major effect

was to increase the $[S_{1/2}]$ ([pyruvate]) (Fig. 6). It is probable, therefore, that the D-aspartic semialdehyde exerts its primary inhibitory effect by influencing pyruvate site cooperativity in some manner rather than simply blocking L-aspartic semialdehyde binding to the enzyme by steric hindrance.

ACKNOWLEDGMENTS

The author is indebted to Mrs. Carolyn Workman and Mr. Denny Carlson for superb technical assistance. This work was supported by National Science Foundation grant number GB-5898 and by National Institute of Health grant number AI 09035 01.

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