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Aspartokinase of *Streptococcus mutans*: Purification, Properties, and Regulation

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Aspartokinase from *Streptococcus mutans* BHT was purified to homogeneity and characterized. The molecular weight of the native enzyme was estimated to be 242,000 by gel filtration. Cross-linking of aspartokinase with dimethyl suberimidate and polyacrylamide gel electrophoresis of the amidinated enzyme in the presence of sodium dodecyl sulfate showed the enzyme to be composed of six identical subunits with a molecular weight of 40,000. The optimal pH range for enzyme activity was 6.5 to 8.5. The apparent Michaelis-Menten constants for aspartate and ATP were 5.5 and 2.2 mM, respectively. The enzyme was stable within the temperature range of 10 to 35°C. Aspartokinase was not feedback inhibited by individual amino acids, but was concertedly inhibited by L-lysine and L-threonine (93.5% inhibition at 10 mM each). The inhibition was noncompetitive with respect to aspartate (K_i = 10 mM) and mixed with respect to ATP. L-Threonine methyl ester and L-threonine amide were able to substitute for L-threonine in feedback inhibition, but the requirement for L-lysine was strict. The feedback inhibitor pair protected the enzyme against heat denaturation. Aspartokinase synthesis was repressed by L-threonine; this repression was enhanced by L-lysine, but was slightly attenuated by L-methionine.

Streptococcus mutans has been demonstrated to be associated with dental caries in experimental animals (18, 19, 25, 26) and in man (16, 20, 27, 42). The unique cariogenic potential of *S. mutans* is closely associated with its ability to form dental plaque, which is dependent upon the synthesis of extracellular polysaccharides from sucrose by enzymatic reactions catalyzed by glycosyl transferases (34, 46).

Even though considerable interests in the nutritional requirements of oral streptococci have developed in recent years (5, 21, 24, 38), perusal of the literature has indicated to us that no studies pertaining to amino acid biosynthesis or its regulation have been conducted in *S. mutans* or any other oral microorganism. Since amino acids present in human saliva have been reported to be insufficient to support optimal growth of lactobacilli or streptococci in the oral cavity (2, 4), and since the amino acid contents of dental plaque are in general known to be very low (9, 23, 31), the synthesis of the needed amino acids and the regulation of this activity must be essential for the survival and proliferation of these microorganisms in the oral environment. Due to our interest in the dental plaque-forming ability and cariogenicity of *S. mutans*, we have chosen to study amino acid biosynthesis and its regulation in this microorganism. We report herein the purification and properties of a regulatory enzyme, aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4), which occupies a strategic position in the biosynthesis of the aspartate family amino acids.

(Part of this work was taken from the M.S. thesis of R.M.M., which was submitted to the University of Maryland).

MATERIALS AND METHODS

Bacterial strain and growth procedures. *S. mutans* strain BHT is a human isolate with a serotype *b*-specific determinant (3). The organism, a gift from A. L. Delisle of our Department, was maintained, with periodic tranfers, on Trypticase soy agar slants containing 0.5% $CaCO_3$. Characterization of the organism was performed by examining colony morphology and its ability to ferment mannitol and sorbitol.

Cultures used for enzyme isolation were grown on 3% Trypticase soy broth in 1-liter batches. Using a 2% fresh inoculum, completion of log-phase growth was achieved at 16 h when incubated at 37°C under stationary conditions. The growth of cultures was monitored with a Klett-Summerson colorimeter (no. 54 filter). Cells were harvested in late log phase (270 to 300 Klett units) and washed twice with cold 0.02 M potassium phosphate, pH 7, containing 0.03 M β-mercaptoethanol and 0.15 M KCl. Cells harvested for repression studies were grown on Carlsson's chemically defined medium (5) and were washed three times with the above buffer to assure complete removal of supplemental amino acids. Culture medium was autoclaved, and amino acids were filter sterilized to avoid possible decomposition.

Preparation of enzyme extracts. Cells were disintegrated by ultrasonic treatment in the presence of micro glass powder as follows. Cell pellets were suspended in 4 volumes of cold 0.02 M potassium phosphate, pH 7, containing 0.03 M β-mercaptoethanol and 0.15 M KCl, along with 2 volumes of 5-μm waterproof glass beads (Heat Systems-Ultrasonics, Inc.). The suspension was treated by 10 30-s exposures to sonic oscillation with a Branson Sonifier at a power setting of 7 A. The temperature of the cell suspension was maintained at 4°C by cooling in a dry ice-alcohol bath. The crude sonic extracts were centrifuged at 15,000 × *g* for 20 min, and the supernatants were stored at −15°C until they were ready to be used.

Enzyme assay. Aspartokinase activity was assayed by measuring the formation of L-aspartic-β-hydroxamate from L-aspartate and ATP in the presence of hydroxylamine according to the procedure described by Stadtman et al. (40). The reaction mixture contained: 20.7 mM ATP, 93.1 mM tris(hydroxymethyl)aminoethane (Tris)-hydrochloride, pH 8.1, 3.1 mM $MgSO_4$, 20 mM L-aspartate, 0.8 M NH_2OH-HCl (freshly neutralized with KOH, thus giving 0.8 N K^+ in the assay), and 50 μl of enzyme preparation in a final volume of 1 ml. After incubation at 29°C for 40 min, the reaction was terminated by adding 1 ml of ferric chloride reagent (10% $FeCl_3$ and 3.3% trichloroacetic acid in 0.7 N HCl). After centrifugation to remove the denatured protein, the optical density of the aspartohydroxamate-iron complex was measured at 540 nm by a Gilford model 2400 spectrophotometer, using β-aspartohydroxamate as a standard.

One unit of aspartokinase activity was expressed as the amount of enzyme required to catalyze the formation of 1 μmol of reaction product per min under the assay conditions described.

Protein concentration was determined by the method of Lowry et al. (32), with crystallized bovine serum albumin as standard.

Enzyme purification. To the 15,000 × *g* supernatant of crude sonic extract (fraction I), 0.1 volume of 10% NaOH-neutralized streptomycin sulfate was added. After 30 min of constant stirring, the precipitated nucleic acid was removed by centrifugation and discarded. The supernatant was dialyzed overnight against 20 volumes of 0.02 M potassium phosphate, pH 7, containing 0.03 M β-mercaptoethanol and 0.15 M KCl. The dialyzed preparation (fraction II) was then fractionated with ammonium sulfate. The fraction precipitating between 35 and 65% saturation, which contained 95% of aspartokinase activity, was dissolved in the above buffer to an appropriate concentration, and was dialyzed against the same buffer (fraction III). Fraction III was passed through a column of Sephadex G-200 (1.5 by 30 cm) previously equilibrated with the above buffer and eluted with the same buffer. The effluent (monitored by optical density at 280 nm) was collected by an automatic fraction collector. Enzyme activity was found in fractions immediately after elution of blue dextran 2000 (monitored at 675 nm). Fractions containing aspartokinase were pooled and concentrated under vacuum to one-tenth of the original volume in a collodion bag (0.008-μm pore size, VWR Scientific Co.) (fraction IV). Fraction IV was treated with calcium phosphate gel (10 mg of gel/mg of protein). The gel suspension was stirred for 1 h and centrifuged. The gel-protein pellet, after four washes with 0.02 M potassium phosphate, pH 7, was eluted stepwise with the same buffer at 0.04 and 0.06 M. Ninety percent of the enzyme activity was eluted at a buffer concentration of 0.1 M. After dialysis in the 0.02 M potassium phosphate, pH 7, the enzyme preparation (fraction V) was chromatographed on a column (0.9 by 60 cm) containing diethylaminoethyl (DEAE)-Sephadex A-50 preequilibrated with 0.02 M Tris-hydrochloride, pH 8.1. The protein was eluted with a linear potassium chloride gradient (0 to 0.54 M KCl) in 1.2 liters of equilibration buffer at a flow rate of 0.5 ml/min. The enzyme was eluted in 50 ml at approximately 0.43 M KCl. Fractions containing enzyme activity were pooled, concentrated, and desalted by membrane filtration (Diaflo; Amicon Corp.). The desalted enzyme preparation (fraction VI) was chromatographed on a Cellex E (Ecteola) column (0.9 by 60 cm) by stepwise elution with 150 ml each of 0.002 M Tris-hydrochloride, pH 8.1, containing no KCl, 0.15 M KCl, and 0.25 M KCl at a flow rate of 0.25 ml/min. The enzyme emerged at the 0.25 M KCl elution in 25 ml. Fractions containing aspartokinase were pooled and concentrated by membrane filtration (fraction VII). All purification procedures were performed at 4°C. A typical purification is summarized in Table 1.

Molecular-weight determination. Molecular weight of the native aspartokinase was estimated by gel filtration on a Sephadex G-200 column, using phosphorylase, aldolase, chymotrypsinogen A, ovalbumin, and ribonuclease A as standard markers and blue dextran 2000 as void-volume indicator. Protein and dextran were detected by absorption at 280 and 675 nm, respectively; aspartokinase was located by enzyme

TABLE 1. *Purification of S. mutans BHT aspartokinase*[a]

Fraction	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
I. Sonic extract	94	3,400	17.3	0.0051	100	1
II. Streptomycin sulfate	91	3,175	17.2	0.0054	99	1
III. $(NH_4)_2SO_4$ (35–65%)	46	950	16.8	0.0177	97	3
IV. Sephadex G-200	12.5	150	14.5	0.0967	84	18
V. Calcium phosphate gel	30	48	9.1	0.1896	53	37
VI. DEAE-Sephadex	30	1.35	7.1	5.26	41	1,031
VII. Cellex E[b]	37.1	0.25	3.48	13.92	20	2792

[a] Starting material was 5 liters of late-log-phase culture.
[b] Ecteola.

assay. The selectivity curve was prepared by plotting K_{av} [defined as $(V_e - V_0)/(V_t - V_0)$, where V_e denotes elution volume for the protein, V_0 elution volume for blue dextran, and V_t total bed volume] against the log molecular weight. In each experimental run, a straight line was obtained by linear regression based on the data of standard marker proteins. The molecular weight of the native aspartokinase was estimated from the selectivity curve by interception at its K_{av} value.

Disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was run on 7% polyacrylamide gel in 0.025 M Tris-glycine, pH 9.5, on a disc gel electrophoresis apparatus (Canalco, Rockville, Md.), following the published procedures of Davis (13) and Ornstein (35). Sodium dodecyl sulfate (SDS)-disc gel electrophoresis was run, in general, according to the procedure of Morimoto and Harrington (33). The concentrated aspartokinase preparation (25 to 175 μg of fraction VII), after dialysis overnight against 0.01 M Tris-glycine, pH 8, containing 0.1% each SDS and β-mercaptoethanol and boiling for 3 min in 10% each SDS and β-mercaptoethanol, was subjected to electrophoresis in the 10% SDS-polyacrylamide gel along with the tracking dye in 10% β-mercaptoethanol and 0.05 M Tris-glycine, pH 8, containing 0.1% SDS. Electrophoresis was conducted at 3 to 5 mA per gel until the tracking dye was 0.5 cm from the bottom of the gel. Gels were stained with Coomassie blue and destained in methanol-acetic acid-water (2:3:35, vol/vol). The standard marker proteins (50 μg each) were also treated and subjected to electrophoresis the same as aspartokinase. The molecular weight of aspartokinase subunits was estimated from the curve prepared by plotting the logarithms of the molecular weights of the marker proteins against their corresponding mobility relative to the tracking dye.

Cross-linking of enzyme and SDS-gel electrophoresis. Cross-linking of aspartokinase was performed, in general, according to the procedure of Carpenter and Harrington (6) and Davis and Stark (12) with some modifications. The enzyme (0.1 mg) was dissolved in 50 μl of 0.2 M triethanolamine-hydrochloride, pH 8.5, containing 0.2 mg of dimethyl suberimidate. After reaction at room temperature for 90 min, 50 μl of 2% SDS–2% β-mercaptoethanol in 0.2 M triethanolamine-hydrochloride, pH 8.5, was added. The reaction was continued at 37°C for 2 h. A 50-μl aliquot of the resulting enzyme solution was mixed with equal volume of 0.027% bromophenol blue in 50% glycerol and was applied to the gel. The gels (13 by 0.6 cm), which contained 5.25% acrylamide, 0.125% methylenebisacrylamide, and running buffer (0.025 M sodium phosphate and 0.5% SDS, pH 8.5), were polymerized with 0.024% ammonium persulfate and 0.06% *N,N,N′,N′*-tetramethylethylenediamine. Electrophoresis was performed at 8 mA per tube. Gels were then removed, stained, and destained as previously described. The molecular weights of the covalently cross-linked protein species were estimated by comparison with standard protein markers by the procedure described above.

Commercially obtained compounds. Dimethyl suberimidate, SDS, amino acids, ATP, and phosphorylase were purchased from Sigma Chemical Co.; streptomycin sulfate from Eli Lilly and Co.; enzyme-grade ammonium sulfate from Schwarz/Mann Research Laboratories; calcium phosphate gel from Calbiochem; bovine serum albumin from Miles Laboratory; DEAE-Sephadex, Sephadex G-200, and marker proteins for molecular-weight determination from Pharmacia Fine Chemicals; disc gel electrophoresis reagents from Canalco; Cellex E (Ecteola) from Bio-Rad Laboratories; and triethanolamine from Fisher Scientific Co. Other reagents and chemicals were of highest purity obtainable from commercial sources.

RESULTS

Purity and stability of enzyme. Enzyme purified to fraction VII gave a major protein-stained band in the polyacrylamide gel cylinders accounting for over 95% of the total protein. Fraction VII, when subjected to electrophoresis on SDS-polyacrylamide gel, also gave a major protein-stained band, presumably the monomeric subunits (see below and Fig. 1). The enzyme, crude or purified, appears to be stable over a period of several months' storage at −20°C. However, during 24 h of storage at 4°C the loss of enzyme activity was substantial, but the enzyme was found to be protected to some degree by β-mercaptoethanol.

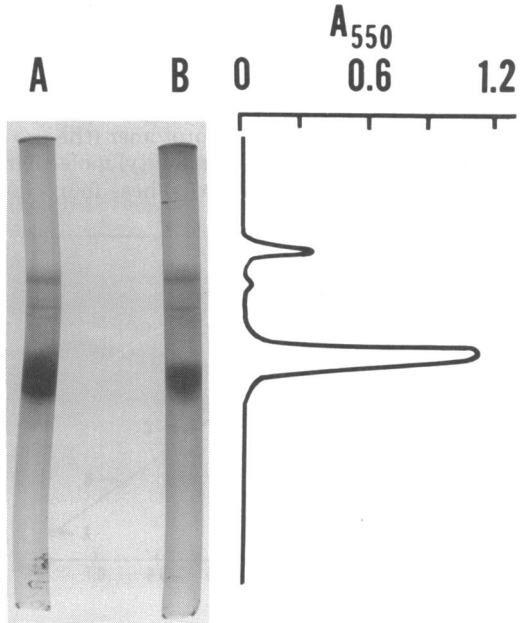

FIG. 1. *SDS-polyacrylamide gel electrophoresis of the purified aspartokinase. (A) 115.2 μg of enzyme (fraction VII) per gel; (B) 28.8 μg of enzyme (fraction VII) per gel. Electrophoresis was carried out at pH 8.0 at 3 mA per tube for about 2 h. The mobility of the aspartokinase relative to the tracking dye was 0.43 for the monomer (lower band) and 0.285 for the presumed dimer (upper band). The spectrophotometric scan (absorbancy at 550 nm [A_{550}]) of gel B was performed on the Gilford linear transport.*

Molecular weight of enzyme and its subunit. The molecular weight of the native *S. mutans* BHT aspartokinase, assuming that it is a globular protein, was estimated to be 252,000 ± 1,400 (mean ± standard deviation, $n = 4$) by Sephadex G-200 gel filtration. A typical run of this experiment is shown in Fig. 2.

SDS-gel electrophoresis of fraction VII yielded essentially one major protein-stained band (Fig. 1), which was estimated to have a molecular weight of 40,000 (average of two separate runs) (Fig. 3). Assuming that the dissociation of the native enzyme is complete under the experimental conditions, it would suggest that aspartokinase of *S. mutans* BHT consists of six subunits with similar molecular weight. More reliable data regarding the subunits of aspartokinase, however, were obtained by cross-linking of the enzyme with the bifunctional reagent and SDS-gel electrophoresis as described below.

The results of cross-linking and SDS-polyacrylamide gel electrophoresis of aspartokinase are shown in Fig. 4. Four major protein-stained bands were visible, with the band having highest mobility in greatest intensity (species A in Fig. 4), trailed by the others in decreasing mobility and band intensity. When the migration of each band with respect to the tracking dye was plotted, the molecular weights of the covalently cross-linked protein species produced by amidination were multiples of the protomer (the protein species with the highest mobility) molecular weight of 40,000 (Fig. 4, right). These four spe-

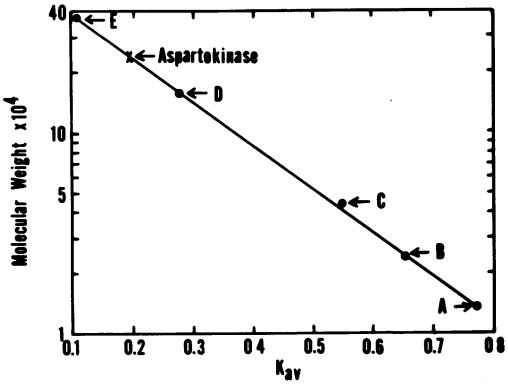

FIG. 2. *Molecular-weight determination of native aspartokinase by Sephadex G-200 gel filtration. The column (2.4 by 40 cm) was preequilibrated and eluted with 0.1 M potassium phosphate buffer, pH 7, with 0.1 N NaCl and 0.02% sodium azide at room temperature. Molecular-weight standards were: (A) ribonuclease A (13,700); (B) chymotrypsinogen A (25,000); (C) ovalbumin (45,000); (D) aldolase (158,000); and (E) phosphorylase (370,000). Twenty milligrams each of the marker proteins and 1.6 mg of fraction VII aspartokinase were applied to the column.*

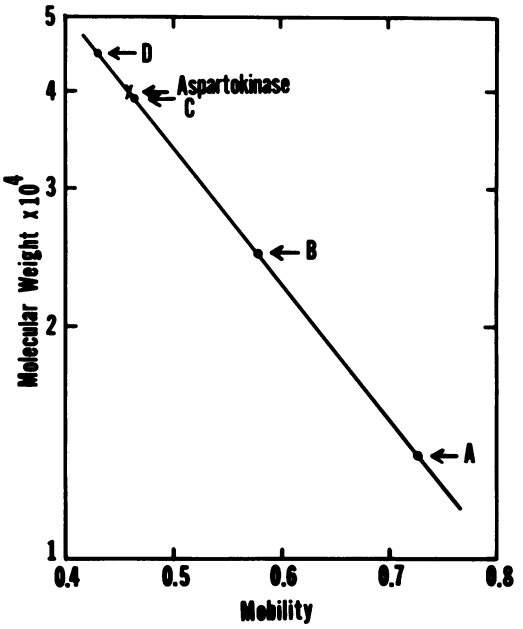

FIG. 3. *Molecular-weight determination of aspartokinase subunits by SDS-polyacrylamide gel electrophoresis. Electrophoresis was performed according to the procedure described in Fig. 1. Molecular-weight standards were: (A) ribonuclease A (13,700); (B) chymotrypsinogen A (25,000); (C) aldolase (38,000); and (D) ovalbumin (45,000).*

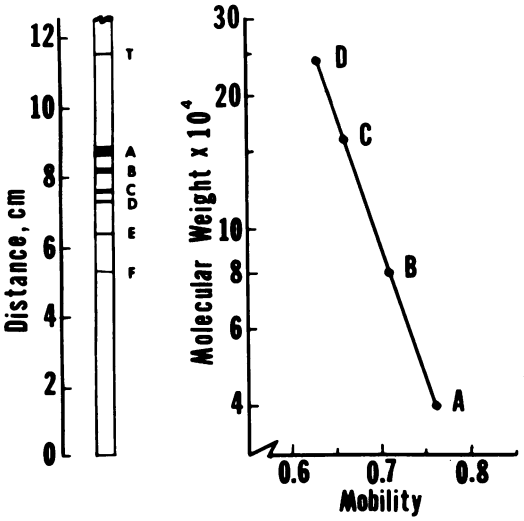

FIG. 4. *SDS-polyacrylamide gel electrophoresis and molecular-weight determination of cross-linked aspartokinase. (Left) Migration of covalently cross-linked protein species on SDS-polyacrylamide gel. (Right) Plot of molecular weight on logarithmic scale of cross-linked protein species against mobility. T, Tracking dye; A, monomer; B, dimer; C, tetramer; D, hexamer; E and F, protein species with molecular weight higher than that of hexamer.*

cies of protein, (A, B, C, and D in Fig. 4) appear to correspond to the monomer, dimer, tetramer, and hexamer of the subunits, respectively. Although species having a molecular weight higher than that of the hexamer protein (E and F, Fig. 4) were also present in the gels in minor amounts, no species corresponding to the trimer or pentamer protein was observed.

Aspartokinase inhibition by amino acids. Amino acids of the aspartate family (i.e., lysine, threonine, methionine, isoleucine, and diaminopimelate) at 10 mM each were found to have little or no effect on the aspartokinase activity of *S. mutans* when they were tested individually. However, the enzyme was subject to strong feedback inhibition by the amino acid pair of L-lysine and L-threonine (93.5% inhibition at 10 mM each); concerted feedback inhibition by L-methionine and L-threonine was also observed, but to a much lesser degree (13% at 10 mM each). No concerted inhibition was observed with L-methionine plus L-lysine (10 mM each). It should be noted, however, that L-methionine displayed slight activation (16%) of enzyme activity when tested alone. Amino acids of other families (L-arginine, L-serine, and L-glutamic acid) at 10 mM each were found to have no effect on enzyme activity.

The sensitivity of aspartokinase to inhibition by increasing concentrations of lysine plus threonine displayed a sigmoidal curve typical of an allosteric enzyme, with maximum inhibition (93.5%) occurring at 10 mM each. The enzyme could not be completely inhibited by further increasing the effector concentrations. The degree of feedback inhibition attainable by this amino acid pair was determined only by the effector at the lower molar concentration, further characterizing the concerted nature of this inhibition (i.e., the requirement for the concomitant presence of both effectors) (Fig. 5).

Enzyme and inhibition kinetics. The substrate saturation kinetics for aspartate and ATP plotted according to the method of Lineweaver and Burk (30) are shown in Fig. 6 and 7, respectively. The V_{max} values were calculated to be 0.357 mmol min^{-1} mg^{-1} for aspartate and 0.372 mmol min^{-1} mg^{-1} for ATP (fraction III, Table 1, was used for this experiment). The values of the apparent Michaelis-Menten constant (K_m) for aspartate and ATP were 5.5 and 2.2 mM, respectively. These results indicate that the enzyme has a slightly greater affinity for ATP than aspartate; similar results were reported by Kuramitsu and Watson (29) for aspartokinase in *Clostridium perfringens*. ATP at high concentrations exhibited substrate inhibition (Fig. 7), but this effect was overcome by the addition of an excess of Mg^{2+} ions in the assay mixture.

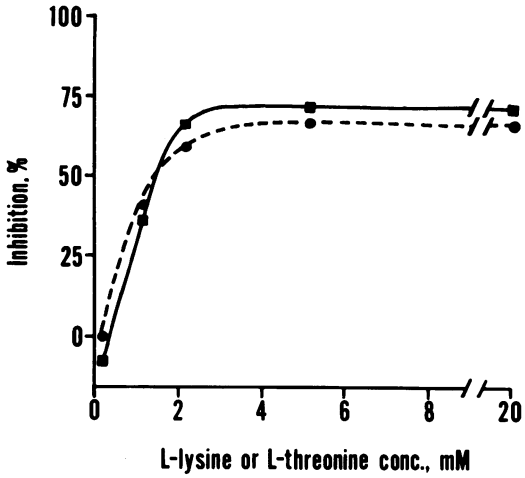

FIG. 5. *Cooperative effect of* L-*lysine and* L-*threonine on aspartokinase feedback inhibition. Symbols:* (●) *Increasing* L-*lysine concentration at 2 mM* L-*threonine;* (■) *increasing* L-*threonine concentration at 2 mM* L-*lysine. Fraction IV aspartokinase was used.*

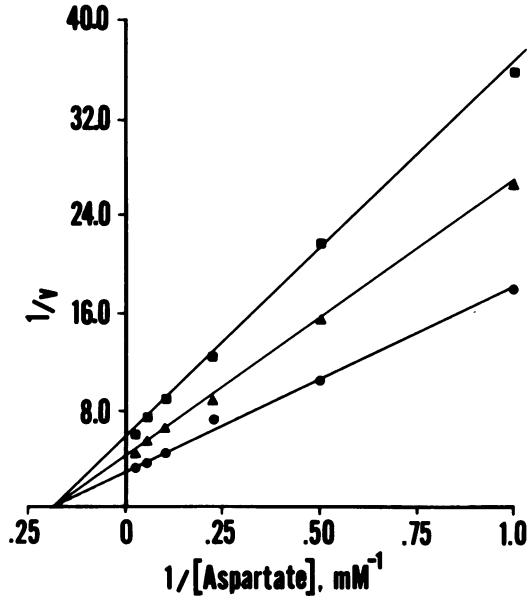

FIG. 6. *Effect of different concentrations of feedback inhibitors on aspartokinase activity as a function of aspartate concentration. Symbols:* (●) *No effectors;* (▲) L-*lysine plus* L-*threonine, 5 mM each;* (■) L-*lysine plus* L-*threonine, 10 mM each. Fraction III aspartokinase was used.*

Both the aspartate and ATP saturation curves were hyperbolic, indicating that the binding of aspartate or ATP to the enzyme is not cooperative. The enzyme had a strict requirement for ATP as a phosphorylating agent; CTP and GTP were not active.

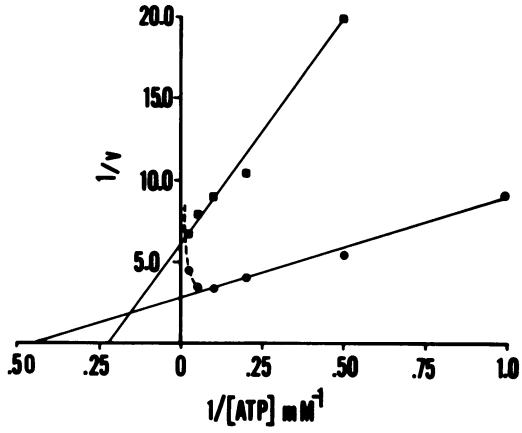

FIG. 7. *Effects of feedback inhibitors on aspartokinase activity as a function of ATP concentration. Symbols:* (●) *No effectors;* (■) L-*lysine plus* L-*threonine, 5 mM each. Fraction III aspartokinase was used. Dashed line depicts excessive ATP effect on aspartokinase activity.*

The feedback inhibitor pair of L-lysine and L-threonine appeared to be noncompetitive with respect to aspartate (K_i = 10 to 11 mM) and exhibited mixed inhibition with respect to ATP. A plot of the kinetic data according to the method of Dixon (14) also bears out the noncompetitive nature of the inhibitor pair on the enzyme with respect to aspartate.

Effect of temperature on enzyme stability and feedback sensitivity. Aspartokinase was relatively stable at temperatures below 35°C, with an optimal activity at 30°C; about 50% of the enzyme was inactivated after incubation for 20 min at 50°C.

Slightly lower concentrations of lysine and threonine were required to attain the same level of inhibition in the heat-treated enzyme. However, no changes in the overall feedback kinetics of the enzyme were observed after heat treatment.

Protection of aspartokinase against heat inactivation by feedback inhibitors. Aspartokinase was protected from heat denaturation by its concerted feedback inhibitors; when the enzyme was heat treated at 50°C for 20 min in the presence of 10 mM each L-lysine and L-threonine, only 13% loss of activity was observed. However, when the enzyme was treated as above in the absence of the two amino acids, 50% of the enzyme activity was lost. Enzyme heat treated in the presence of L-threonine plus L-methionine (5 mM each) under the above conditions lost about 30% activity. The enzyme substrates, aspartate and ATP (20 mM each), or the effectors, L-lysine and L-threonine (10 mM each), individually were found to provide no significant protection for the enzyme against heat inactivation.

Effect of pH on catalytic and regulatory properties of aspartokinase. Optimal aspartokinase activity was observed over a broad pH range of 6.5 to 8.5 (Fig. 8). In pH regions below 6 and above 9, the enzyme activity rapidly decreased. The level of inhibition of aspartokinase by the inhibitor pair lysine and threonine examined at various pH conditions indicates that aspartokinase was less inhibited at the pH outside the optimal pH range for the enzyme (Fig. 8).

Effect of growth media on aspartokinase synthesis. When L-threonine (this and other amino acids tested were at 10 mM each) was present in the growth medium, a 41% decrease in the specific activity of aspartokinase was observed. Although L-lysine alone was stimulatory (22%) on aspartokinase synthesis, it slightly enhanced the repressive effect of L-threonine (51%). Lysine plus threonine thus seem to both inhibit aspartokinase activity and repress its synthesis. The effect of these two amino acids was not additive, nullifying the possibility that their modes of action are independent of one another.

L-Methionine, L-isoleucine, and diaminopimelic acid, when added to growth medium individually or in combination with L-lysine, produced either no effect or insignificant enhancement of enzyme synthesis. However, when L-

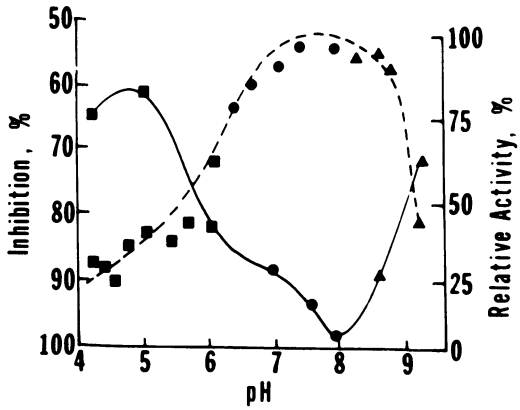

FIG. 8. *Effect of pH on the catalytic activity and sensitivity to feedback inhibition of aspartokinase. Enzyme activity was measured in the presence of 10 mM each* L-*lysine plus* L-*threonine (solid line for percent inhibition) or in the absence of effectors (dashed line for relative activity). Buffers used were:* (■) *potassium biphthalate-NaOH;* (●) *potassium phosphate; and* (▲) *Tris-hydrochloride. Hydroxylamine was neutralized with KOH to the pH values of the buffers used. Fraction III enzyme preparation was used.*

methionine was combined with L-threonine, it attenuated the repressive effect of L-threonine (16%). Aspartokinase obtained from cultures grown in the presence of L-lysine and/or L-threonine showed no observable differences in its feedback regulatory characteristics as compared with the nonsupplemented culture.

Structural specificity of feedback inhibitors. Individually, none of the L-threonine analogs (2 mM each) tested, i.e., D-threonine, L-threonine methyl ester, L-threonine amide, glycyl-L-threonine, and *N*-methyl-L-threonine, was inhibitory to aspartokinase, but when tested in the presence of L-lysine at a 1:1 molar ratio, L-threonine amide and L-threonine methyl ester were able to replace L-threonine to a significant extent (approximately 50% efficient) for concerted inhibition. Neither the N-substituted L-threonine (i.e., glycyl-L-threonine and *N*-methyl-L-threonine) nor the D-isomer (i.e., D-threonine) was an active analog.

L-Lysine analogs (2 mM each), i.e., D-lysine, ornithine, α-aminoadipate, cadaverine, *meso*-, LL-, and DD-diaminopimelate, L-cysteine, and L-arginine, tested either alone or in combination with L-threonine, did not give any appreciable inhibition on aspartokinase.

Effect of growth phase and degree of purification on aspartokinase activity and regulation. Aspartokinase levels in *S. mutans* BHT cells examined at various phases of growth showed only slight variation in enzyme levels throughout the course of growth (specific activity 0.038, 0.046, 0.047, and 0.042 U/mg for early log, mid-log, late log, and stationary phases, respectively). The concerted feedback inhibition by lysine and threonine, examined in cells from various growth phases, showed no significant differences. Enzyme purified to various degrees did not show any alteration in the feedback inhibition kinetics.

DISCUSSION

The purification scheme utilized in this study yielded approximately 2,800-fold purification of aspartokinase from *S. mutans* BHT, with 20% recovery (Table 1). The most purified enzyme preparation (fraction VII, Table 1) was judged to be over 95% pure. This preparation was therefore used for molecular-weight determinations. The molecular weight of the native aspartokinase of *S. mutans* BHT was found to be 242,000. Results obtained from SDS-gel electrophoresis suggest that the enzyme may consist of subunits having a molecular weight of 40,000. However, information regarding the subunit structure of aspartokinase was obtained from a more rigorous study by cross-linking of the enzyme with the bifunctional reagent dimethyl suberimidate and SDS-gel electrophoresis (6, 12). A plot of the electrophoretic mobility of the cross-linked protein species against the logarithm of molecular weight fell on a straight line for mobility between 0.6 and 0.8 (Fig. 4). Since the predominant protein species has the lowest molecular weight, i.e., 40,000, and the other covalently cross-linked protein species have molecular weights that are multiples of 40,000, it can be assumed that aspartokinase of *S. mutans* BHT is composed of six identical subunits, each with a molecular weight of 40,000. This observation is consistent with the allosteric behavior of this enzyme as presented in this study.

It is notable that the cross-linked protein species with molecular weight corresponding to the monomer, dimer, tetramer, and hexamer were present, but no protein species of the trimer or pentamer was visible in the SDS-gels from several experimental runs. The data suggest that the six subunits of the enzyme may be arranged in a form of octahedral structure, which favors cross-linking at one interface over the other, giving rise to the dominance of the dimer, tetramer, and hexamer over the trimer and pentamer (see reference 6 for discussion on this subject). The present data on *S. mutans* aspartokinase, however, were not obtained in sufficient detail to propose a definitive quaternary structure for this enzyme.

The regulatory importance of feedback inhibition by end products on the activity of an early enzyme in a biosynthetic pathway has been well documented (10, 39, 44). In bacteria, aspartokinase is responsible for the first reaction in the biosynthesis of the amino acids of the aspartate family: lysine, threonine, methionine, and isoleucine. In *S. mutans,* lysine plus threonine were simultaneously required to exert a concerted type of inhibition on aspartokinase. This effect was quite specific: no other amino acids except methionine, either in pairs or alone, had any appreciable inhibitory effect. The peculiar behavior of methionine on aspartokinase activity, i.e., slight activation when tested alone but slight inhibition when combined with L-threonine, may reflect an additional mode of regulation by this pathway end product. The dual regulatory role of L-methionine in *S. mutans* aspartokinase is analogous to that reported for the enzyme from other organisms (15, 17, 37).

The sigmoidal curve demonstrated with increasing concentrations of the feedback effectors, lysine and threonine, indicates that multiple effector sites probably exist on the enzyme and act in a cooperative fashion. The requirement of a concomitant presence of L-lysine and L-threonine for feedback inhibition (Fig. 5) indicates the concerted manner of this feedback

inhibition. Concerted inhibition of aspartokinase by lysine and threonine has previously been shown in other organisms (15, 22, 28, 36, 41). In other bacteria, purification of aspartokinase has been shown to enhance inhibition by L-lysine and L-threonine (15, 28). In *S. mutans* aspartokinase, however, the purification procedures used did not cause any change in feedback sensitivity. This fact, along with the observation that lysine and threonine did not independently inhibit the *S. mutans* aspartokinase, indicates that this enzyme is unlikely under the isoenzymatic type of feedback control.

The fact that lysine and threonine, when present together, protect the enzyme from heat inactivation is a common phenomenon also observed with other feedback effectors for other regulatory enzymes (7, 11, 28, 40). Protection of the enzyme against heat denaturation is probably due to the conformational change induced by the binding of these two allosteric effectors. Individually, the inhibitors lacked any protective effect against heat inactivation, strongly indicating the cooperative nature of these two effectors. Since lysine and threonine at the same concentration levels exhibited a greater degree of inhibition on the heat-treated enzyme, heat treatment appears to enhance the binding of the effectors to *S. mutans* aspartokinase.

Aspartokinase from *S. mutans* BHT exhibited a broad pH range for optimal activity. Concerted inhibition of the enzyme by lysine and threonine was highest within the optimal pH range of the enzyme (Fig. 8), suggesting that the enzyme configuration that favors catalysis also favors the binding of the feedback effectors. Since none of the pK values or isoelectric points of lysine and threonine fall near pH 8, where the maximum inhibition of enzyme activity was observed, the influence of pH on the degree of inhibition may be related to the ionic state of the enzyme. However, with the present data it is not possible to speculate on the ionic state of the enzyme in reference to its interaction with the effectors.

Examination of the structural specificity of the amino acid effectors revealed that the α-carboxyl group of L-threonine was not required for concerted inhibition when paired with L-lysine; however, the freedom and configuration of the amino group appears to be critical. None of the lysine analogs tested with L-threonine displayed concerted inhibition on aspartokinase, indicating a higher degree of structural specificity for L-lysine as a coeffector.

Most amino acids, including lysine and threonine, are found to be present in dental plaque at very low levels (1, 9, 23, 31). The amino acid requirements of *S. mutans* have been studied by various investigators (8, 43, 45). Cystine and glutamate were found to be required by *S. mutans* BHT under anaerobic growth conditions (with carbonate); when grown aerobically (with no carbonate), arginine, leucine, and isoleucine were required in addition to cystine and glutamate. It was noted that neither lysine nor threonine was required for growth under either aerobic or anaerobic conditions (43). Besides the present investigation, no other studies have been reported concerning the biosynthesis and its regulation of other amino acds in *S. mutans*.

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