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Glutamine Synthetase of *Bacillus stearothermophilus.* I. Purification and Basic Properties†

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ABSTRACT: To assess the amount of complex functional information that has evolved and is maintained in a thermophilic regulatory enzyme, glutamine synthetase from *Bacillus stearothermophilus* has been isolated and partially characterized. Enzyme synthesis is derepressed by limiting ammonia or glutamine in chemically defined media at 55°. Procedures for purification of the enzyme to homogeneity are described, including an affinity gel chromatography step. Polyacrylamide gel electrophoresis with sodium dodecyl sulfate indicates a subunit of mol wt ≃51,000, and electron microscopy reveals a double hexagonal dodecameric subunit arrangement. The enzyme resembles that from *Bacillus subtilis* in amino acid content and lack of bound cofactors or adenylyl groups. Activity is stimulated by bound metal ions, $Mn^{2+} > Mg^{2+} \simeq Cd^{2+} > Co^{2+} >$ others, each of which induces a different pH-activity profile. Optimal activity occurs at $[Mn^{2+}] = [ATP]$. Binding constants and specificity for substrates at 55° are comparable to those for mesophilic enzymes. The plot of velocity *vs.* [Mn-ATP] is sigmoidal with Hill $n = 2.3$, whereas those for NH_3 and L-glutamate are hyperbolic. With saturating substrate levels, the Arrhenius plot is nonlinear, concave downward with an intersection point at 55°. These phenomena appear to indicate multiple enzyme conformational states.

Glutamine synthetase catalyzes the reaction

$$ATP + \text{L-Glu} + NH_3 \rightarrow \text{L-Gln} + ADP + P_i \quad (1)$$

Its activity is highly regulated in microorganisms by end-product metabolite feedback inhibition, since it occupies a key position in nitrogen metabolism (Shapiro and Stadtman, 1970). The present study is part of an investigation to determine how much of the complex functional information of the mesophilic enzymes has evolved and is maintained in thermophiles.

Despite extensive research on thermophilic proteins, the molecular basis for thermostability is not yet well defined. As reflected by recent reviews of this subject (Singleton and Amelunxen, 1973; Amelunxen and Lins, 1969; Howell *et al.,* 1969), the contributing factors appear to be both various and subtle.

In this paper we describe a procedure for preparing and purifying to homogeneity the glutamine synthetase from *Bacillus stearothermophilus.* This includes a new affinity gel method, using a glutamate side-arm ligand. The basic physicochemical and kinetic properties are reported. These results provide the basis for more detailed mechanistic studies. The accompanying paper (Wedler and Hoffmann, 1974) deals with our investigations on the regulation of enzyme activity and those forces lending thermostability to the protein.

Experimental Section

Materials. All biochemicals used were of the highest purity obtainable from Sigma Chemical Co. Proteins were from Sigma and Worthington. Inorganic salts and metal ions were of analytical grade from Fisher Scientific. A culture of *B. stearothermophilus,* strain 4S, was kindly supplied by Dr. Neal E. Welker, and was transferred every 6-8 weeks to 2% trypticase-agar (BBL) slants, grown at 55°, and stored at 4°.

Growth Media. Cells were grown first in a rich preinoculation broth, collected by centrifugation, washed with "M" buffer (Welker and Campbell, 1963), and transferred to minimal media, which contained (in g/10 l.) 57.6 glucose, 0.53 NH_4Cl, 1.05 L-Arg, 0.6 D,L-Met, 1.44 D,L-Val, 1.5 thiamine, 0.015 nicotinic acid, 0.00001 *d*-biotin, 5 potassium acetate, 10 KH_2PO_4, 10 NaCl, and 0.05 each of $FeCl_3 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, and $CaCl_2 \cdot 6H_2O$. Initially, starter cultures of the limiting medium were supplemented with 0.1% casein-amino acids, to reduce lag times in growth curves. Each volume was transferred to a

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10× larger one, up to 200-l. volumes. Yields of cells in limiting media were *ca.* 1 g/l. Aeration was not a critical growth parameter. Cells were harvested at 1–2 hr into stationary phase by continuous-flow centrifugation.

Enzyme Assays. One unit of enzyme activity is defined as that yielding 1 μmol of product/mg of protein in 5 min at 55° under optimal assay conditions. Two assays were used: each contained (in mM) 50 imidazole, 7.5 ATP, 7.5 $MnCl_2$, 50 L-Glu, and 50 NH_4Cl. (a) *Forward transferase* (for use with impure enzyme preparations): carried out in 0.4 ml at pH 6.5 with 50 mM NH_2OH substituted for NH_4Cl. The reactions (usually 1.0 ml) were stopped with 2.0 ml of acidic $FeCl_3$ solution (Shapiro and Stadtman, 1971); 1 μmol of γ-glutamylhydroxamate (Glu-NHOH)[1] gave A_{540} = 0.274. Control reactions omitted ATP as a check for nonspecific Glu-NHOH synthesis. (b) *Biosynthetic* (for use with purified enzyme): carried out in 0.2-ml total at pH 6.0. The $FeSO_4$-molybdate stop solutions of Shapiro and Stadtman (1971) were used; 1 μmol of P_i released gave A_{660} = 1.60. Control reactions omitted L-Glu as a check for ATPase activity. The two assays give linear responses over the ranges of 0–3.50 and 0–0.35 μmol of product, respectively.

Thermostating of assay mixtures at 55 ± 0.2° was accomplished with a Techne Dri-Block. Protein concentrations were assayed by the method of Lowry *et al.* (1951) or at 280 nm (see Results).

Enzyme Purification. All steps were carried out at 4° unless specified otherwise. Centrifugations were routinely at 20,000*g*.

1. CRUDE EXTRACT. Frozen cells were thawed and suspended in buffer A (50 mM imidazole-acetate–1 mM $MnCl_2$, pH 7)[2] with 2 ml of buffer/g of cells; 50-ml portions were sonicated for 10 min each with cooling. Alternatively, continuous flow could be used for larger cell slurry volumes, with sonication for 15 min/50-ml cells and a flow rate to allow three passes of the volume in the total time. Broken cells were centrifuged at 14,000*g* for 30 min. After adjustment of the protein concentration of the supernatant to 20–25 mg/ml with buffer A, streptomycin sulfate (10% w/v) was added to a final (v/v) ratio of 10% (final concentration = 1%). After stirring 15 min, the solution was then centrifuged at 14,000*g*. To the supernatant was added (with extreme caution in an efficient fume hood) 1 ml/l. of diisopropyl fluorophosphate. The solution was stirred at room temperature for 30 min, then dialyzed at 4° against two changes of 10 volumes of buffer A over 24 hr, then recentrifuged.

2. AMMONIUM SULFATE FRACTIONATION. At 4°, solid ammonium sulfate was added to the supernatant to 60% saturation (di Jesco, 1968), stirred 30 min, then centrifuged at 20,000*g*. The pellet was resuspended, triturated, and stirred in a volume of buffer A equal to the original, with 50% saturated ammonium sulfate for 60 min, then centrifuged at 20,000*g*. This extraction was repeated with an equal volume of A with 47% saturated ammonium sulfate (then 45%, then 40%, if necessary) until 90% of the original activity units were extracted. The supernatant were then combined and the per cent saturation of ammonium sulfate was raised to 80% with solid ammonium sulfate. The solution was stirred 15 min, then centrifuged. The pellet was resuspended in one-fourth the original volume of buffer A from step 1, triturated at pH 6.5, then dialyzed at once using a hollow fiber device (Bio-Rad HFD-1) against two 10× volumes of buffer A.

3. ACETONE PRECIPITATION. Protein concentration was adjusted to 5–10 mg/ml with buffer A and the pH to 5.05 with 1.0 M acetic acid. Reagent grade acetone was added slowly with rapid stirring at 25° to 28.5% (v/v) and the mixture was stirred for 5 min, keeping the observed pH at 5.05. The solution was then centrifuged at 20,000*g* for 5 min and the pellet was rapidly resuspended in one-tenth the original buffer volume. After trituration and stirring for 30 min, the slurry was recentrifuged and the pellet was discarded. The supernatant was dialyzed against 10 volumes of buffer A and then concentrated on a collodion bag device (Sartorius SM-132).

4. AGAROSE 0.5m FILTRATION. The protein solution was applied to a 5 × 80 cm column of Bio-Gel A-0.5m, preequilibrated with buffer A (pH 6.5), and then eluted with A. Enzyme activity emerges in the excluded protein peak.

5. DEAE-CELLULOSE CHROMATOGRAPHY. A column of Whatman DE-52 was packed (4 × 30 cm, or about 5-ml bed volumes/mg of protein) and equilibrated with 10-fold diluted buffer A (pH 7.5). Protein was applied and the column was washed with an amount of A equal to several bed volumes or until $A_{280} < 0.05$. Then a gradient of 10× diluted A *vs.* the same solution with 0.5 M KCl (pH 7.5) was begun, with the volume in each reservoir being 3–5 bed volumes of the column. Enzyme was eluted at about 0.3–0.4 M KCl. The most active fractions were pooled, concentrated, and then dialyzed, rapidly in ultrafiltration and dialysis hollow fiber devices (Bio-Rad HFD-1 and HFU-1), respectively, against 10 mM imidazole-acetate.

6. AFFINITY GEL CHROMATOGRAPHY. Glutamate-derivatized Agarose 1.5m was prepared as described below, packed into a 1 × 10 cm column, and then equilibrated with 10 mM imidazole-acetate (pH 6.5). Enzyme from the previous step was applied at 2–10 mg/ml, the column was washed with 50 ml of equilibration buffer, then a gradient of 25 ml of equilibration buffer *vs.* the same buffer with 0.2 M L-glutamate was begun. Enzyme emerges at 0.05–0.10 M glutamate.

Notes on the Purification. Enzyme is not stable for more than a few hours in solution of very low or very high ionic strength at 4°. Optimal $\mu \simeq 0.1$ M. Diisopropyl fluorophosphate addition inhibits some proteolytic enzyme activity not removed until the Agarose filtration step. Mn^{2+} appears to lend stability in the first three steps. Thiols do not appear necessary for stabilization during purification or for long-term storage at 4°.

If high specific activity (>1.0 U/mg) is obtained in the original cell extract, the above procedures may suffice to purify the enzyme to homogeneity. If adequate per cent purifications are not obtained in each step or if low specific activity enzyme is obtained initially, the following additional steps may be used between steps 5 and 6.

S-1. Calcium Phosphate Cellulose Chromatography. A column of calcium phosphate, precipitated onto microgranular cellulose (Bio-Rad Cellex MS), as described by Koike and Hamada (1971), was packed to 4 × 8 cm and equilibrated with 10 mM phosphate buffer (pH 7.3) (B). Protein was applied and the column washed with 100–200 ml of B until $A_{280} < 0.05$. Then a gradient of 400 ml each of B *vs.* 0.20 M KH_2PO_4 (pH 7.3) was begun. Enzyme activity emerges at *ca.* 0.05–0.06 M phosphate.

S-2. Agarose 1.5m. Protein was then concentrated to an appropriate volume (*ca.* 5 mg/ml of protein) and applied to a 3 × 80 cm column of Agarose 1.5m, equilibrated with buffer A (pH 6.5). Fractions of 5 ml or less were collected so as to isolate the enzyme, which emerges in the leading edge of the excluded peak. Tubes of maximal specific activity were pooled and reconcentrated.

[1] Abbreviation used is: Glu-NHOH, γ-glutamylhydroxamate.

[2] Buffer A is used in different steps of the purification at different pH values.

As an additional procedure, if needed, preparative electrophoresis at pH 7.5 with a Bio-Gel P6 support medium (Whitehead *et al.*, 1971) was quite effective. Once purified, the enzyme is stable for weeks in pH 7.0 buffer, 0.02 M imidazole-acetate, with KCl added to give an ionic strength of 0.1 M.

Ultracentrifugation. Protein was sedimented at 25 ° in buffer A on a Spinco Model E ultracentrifuge, equipped with RTIC and schlieren optics with an Eastman 778 filter. Sedimentation velocity was observed with bar angles of 60°. A plot of ln r *vs.* time gave an observed sedimentation coefficient, which was then corrected to $s_{20,w}$.

Electrofocusing. Protein (0.6 mg) was placed in the 9th tube of 24, comprising a gradient of sucrose (40–0%) over the pH range of 4.0–6.0, using LKB Ampholine carrier ampholites. Each was layered into an LKB 8100-10 column and focusing was carried out at 6° for 24 hr at 350 V. Fractions of 1.5 ml were collected, the pH of each was carefully read, and both protein and activity were assayed.

Amino Acid Analysis. Analyses were carried out on *ca.* 200-μg quantities of protein using a Durrum D-500 instrument, with samples taken at 24, 48, and 72 hr during hydrolysis in 6 N HCl, 110°. Amino acid levels were calculated as the number of residues per 51,000 molecular weight units.

Electrophoresis. Disc gel electrophoresis was carried out with 3.5% gels according to Brewer and Ashworth (1969). Duplicate gels were stained with Coomassie Blue and activity was visualized by the technique of Lee and Dougall (1972). Studies with sodium dodecyl sulfate were with 5% gels (Weber and Osborn, 1969).[3]

Electron Microscopy. Protein (30 μg/ml) was applied to 200 or 400 mesh copper grids and negatively stained with uranyl acetate as described by Dickson *et al.* (1973) except that the parlodian film was not dissolved away. T4 phage were included as dimension standards. Micrographs were obtained using a Siemens Elmiskop 1A instrument, set at an accelerating voltage of 80 kV, and images were recorded on Kodalith LR 70-mm roll film. Assuming a 940-Å length for T4 tail sheath (Eiserling and Dickson, 1972), a Nikon microcomparitor was used to calculate molecular dimensions. Standard differential error analysis of observed dimensions was carried out.

Affinity Gel Preparation. Packed Bio-Rad Agarose 1.5m (100 ml) was derivatized with CNBr (30 g) in 100 ml of H_2O, according to the procedures of Cuatrecasas *et al.* (1968) and Cuatrecasas (1970). After 12-min reaction at 20°, pH 11, the gel was rapidly filtered with suction and washed on the Büchner funnel with ice-water after which 100 ml of cold 2 M hexamethylenediamine (pH 10) was rapidly mixed with the gel and then stirred for 16 hr at 4°. After washing the gel, 0.1 mol of succinic anhydride in 100 ml of cold water was added immediately and stirred at 4°, maintaining pH 6.0 by addition of 2% NaOH until the pH did not change, usually 12–15 min. The carboxylate side-arm group was then activated by dropwise addition of 0.5 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Aldrich) in 30 ml of H_2O to the washed gel in 50 ml of H_2O at pH 4.7, 25°. Then 100 ml of 1 M L-glutamate was added and the mixture was stirred at 25° for 20 hr. Finally, the gel was washed and stored in distilled water at 4°.

Results

Cell Growth and Repression of Enzyme. The effects of using different carbon and nitrogen sources on the extent of cell growth and specific activity of glutamine synthetase are shown in Table I. The choice of C source has little effect, but the N source chosen is obviously important. High levels of NH_4Cl or glutamine can dramatically repress enzyme synthesis, but glutamate is much less effective. Also, supplementation of the minimal medium with Bacto-tryptone or casein-amino acids stimulates cell growth but lowers specific activity markedly. Optimal specific activity was found for cells grown with 1.0 mM NH_4Cl and 20–30 mM dextrose. Specific activity was optimal and constant from mid-log phase and for several hours into stationary phase. Cells were routinely grown on the minimal medium described in the Experimental Section and harvested 2 hr into stationary phase, after chilling with ice to 10° or below.

TABLE I: Effect of Carbon and Nitrogen Sources on Glutamine Synthetase in *B. stearothermophilus*.[a]

	Concn (mM)	Cell Density[d]	Sp Act. (U/mg)
	A. C Source[b]		
Dextrose	20	0.45	2.5
Glycerol	20	0.36	3.0
Sucrose	10	0.70	2.2
	B. N Source[c]		
NH_4Cl	1.0	0.90	4.0
	2.5	0.90	3.1
	5	0.87	1.8
	10	0.58	0.25
	20	0.52	0.10
L-Gln	2.5	0.52	1.1
	10	0.68	0.2
	20	0.82	0.1
L-Glu	5	0.46	0.9
	10	0.52	0.8
	20	0.45	0.5

[a] Specific activity determined by biosynthetic reaction, with controls, on crude cell extracts carried through step 1 of purification scheme (Table II). [b] With 2.5 mM NH_4Cl. [c] With 20 mM glucose. [d] Optical density at 660 nm.

Enzyme Purification. The results of a complete purification, with supplementary procedures included (see Experimental Section), are summarized in Table II. Depending upon growth conditions, cells may have a higher initial specific activity (up to 4 U/mg of protein) in step 1. Purification also apparently removes some inhibitory materials, often giving a greater number of units in steps 3–5 than are observable in earlier steps. Yields from this multistep procedure are quite satisfactory. The affinity gel step serves mainly to remove several minor contaminants not otherwise easily separated from the enzyme. This is not an efficient step with less pure fractions, since glutamate binding is apparently mainly ionic in nature, and buffer with ionic strength above 10–20 mM or high concentrations of other proteins can prevent specific and efficient enzyme binding.

Tests for Homogeneity. Purified protein was subjected to a variety of tests to verify the homogeneity of the preparation. Figure 1 shows the results of sedimentation velocity and polyacrylamide disc gel electrophoresis experiments. The completely symmetrical peak of Figure 1A indicates a lack of significant quantities of impurities with sedimentation behavior dif-

[3] Proteins used as standard markers included bovine serum albumin, rabbit muscle pyruvate kinase, *Escherichia coli* glutamine synthetase (E_{1-7}), *E. coli* aspartate transcarbamylase (R and C. subunits), and δ-chymotrypsin.

TABLE II: Purification of Glutamine Synthetase from *B. stearothermophilus*.[a]

Step	Vol (ml)	Protein (mg/ml)	Sp Act. (U/mg)[b]	Yield (%)
1. Crude extract	318	15.9	0.66	(100)
2. $(NH_4)_2SO_4$ fraction	136	15.6	1.14	73
3. Acetone precipitate	100	7.3	3.16	79
4. Agarose 0.5m	238	2.3	7.12	117
5. DEAE-cellulose	180	1.6	20.6	181
S-1. Ca–P chromatography	16	4.0	42.0	82
S-2. Agarose 1.5m	7.0	2.0	103	47
6. Affinity gel	5.4	2.5	160	25

[a] From 100-g wet packed cells; see Experimental Section for procedures. [b] Specific activity, standardized to the biosynthetic activity levels used in steps 5–6.

ferent from the glutamine synthetase. The photograph shown is one of seven obtained during this experiment; in none of these did the schlieren peak show any asymmetry.

Figure 1B indicates that the purification scheme outlined in Table I leads to an electrophoretic pattern at pH 8.9 with a single major band. Activity also corresponded exactly with this band, as visualized by a transferase assay procedure (Lee and Dougall, 1972). Protein was also denatured with sodium dodecyl sulfate and then electrophoresed, as described in Figure 2A and the Experimental Section. A single band appeared upon staining with Coomassie Brilliant Blue G-250.

Isoelectric focusing indicated a single peak for both activity (transferase) and protein (A_{280} and Lowry techniques) and that the specific activity was constant throughout. All these criteria, taken together, indicate an essentially (>99%) homogeneous protein preparation. The isoionic pH is 4.6.

Structure and Molecular Weight. The purified native enzyme (7.8 mg/ml) was subjected to ultracentrifugation in a single sedimentation velocity experiment, one schlieren pattern of which is shown in Figure 1A. A value for $s_{20,w}$ of 19.75 S was calculated through a sedimentation velocity experiment with the purified enzyme (7.8 mg/ml). Under comparable conditions the glutamine synthetases from *Bacillus subtilis* and

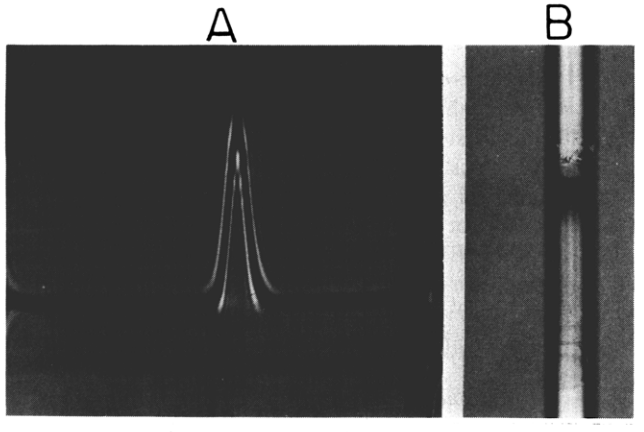

FIGURE 1: Tests for homogeneity of purified *B. stearothermophilus* glutamine synthetase. (a) Schlieren pattern, photographed 40 min after reaching 44,770 rpm in a sedimentation velocity experiment. The solution contained 7.8 mg/ml of enzyme from step 6 of Table I in buffer A (see Experimental Section). (b) Polyacrylamide (3.5%) disc gel electrophoresis of purified enzyme from step 6 of Table II.

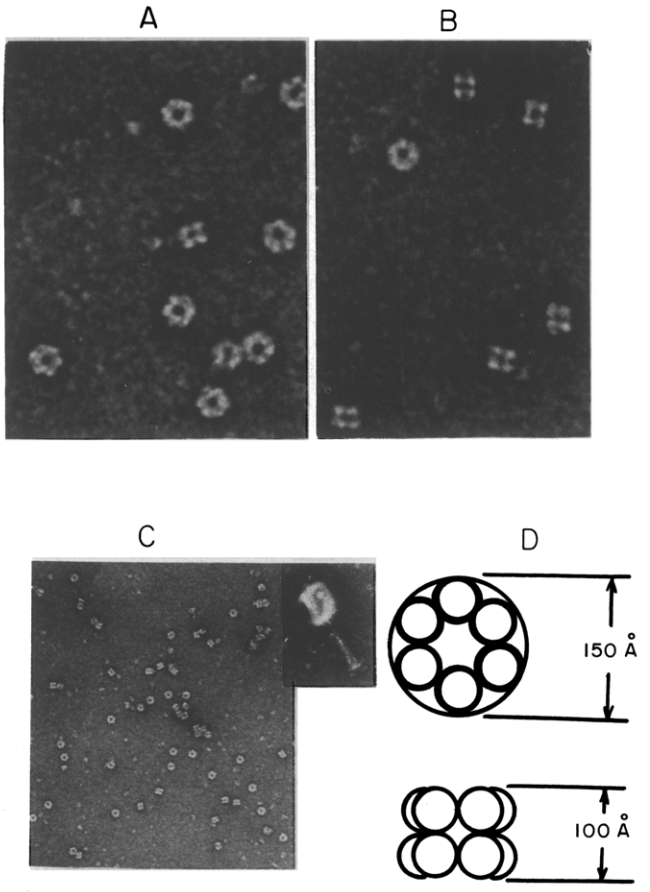

FIGURE 2: Electron micrographs of purified *B. stearothermophilus* glutamine synthetase, showing (A) mainly top views of apparently hexagonal structures, (B) mainly side views, apparently of stacked hexagons, and (C) both top and side views, as well as some clustering of dodecameric structures, as applied to the grids (see Experimental Section). Magnification was 528,000 × for A and B, and 132,000 for C in these photographs. (D) Dimensions of the dodecamer, estimated from the length of T4 phage tail sheath included as an internal dimension standard. (See inset of C.)

Escherichia coli have $s_{20,w}$ values of 18.1 and 19.3 S, respectively (Deuel *et al.*, 1970; Shapiro and Ginsburg, 1968).

Electron microscopy was carried out to determine size, shape, and subunit arrangement of native enzyme. Typical resultant photographs are shown in Figure 2 at several levels of magnification. The pictures show unstained areas with shapes that strikingly resemble those observed previously by Valentine *et al.* (1968) and Deuel *et al.* (1970) for the *E. coli* and *B. subtilis* enzymes, respectively. Figure 2A shows several apparently hexagonal arrangements of subunits, whereas Figure 2B illustrates several side views of dimers of these hexagonal arrays, stacked in an eclipsed fashion. It is noticeable, but perhaps not significant, that *ca.* ⅓–½ of the native dodecamers is observed in close juxtaposition to each other on the grid, rather than distributed randomly and evenly over the surface (Figure 2C). None of the highly polymerized structures observed by Valentine *et al.* (1968) with the *E. coli* enzyme were seen under these conditions, however. T4 phage were added and used as internal dimension markers in these experiments. From the length of T4 tail sheath, the glutamine synthetase dimensions were estimated to be 150 ± 4 Å for the diameter of the hexamer, and 100 ± 4 Å for the thickness of the stacked dodecamer, shown in Figure 2D. These dimensions are about 10% larger than those reported for the *E. coli* or *B. subtilis* proteins (Valentine *et al.*, 1968; Deuel *et al.*, 1970).

Finally, native enzyme, denatured in sodium dodecyl sulfate,

TABLE III: Amino Acid Composition of *B. stearothermophilus* Glutamine Synthetase, Compared to Analyses of Enzymes from Other Sources.[a]

Amino Acid	*B. stearothermophilus*	*B. subtilis*[b]	*E. coli*[c]	Ovine Brain[d]	Pea Seed[e]
Lys	26	29	26	25	26
His	10	12	16	11	8
Arg	26	23	25	25	19
Asp	48	44	47	41	42
Thr	23[f]	20	18	20	23
Ser	23[f]	20	26	25	26
Glu	50	51	43	44	41
Pro	32	29	25	25	19
Gly	32	26	36	41	26
Ala	40	40	42	29	29
½-Cys	10[g]	4	5	12	2
Val	28[h]	26	27	18	27
Met	13	12	15	11	6
Ile	29[h]	26	22	21	23
Leu	40	37	30	22	40
Tyr	13	16	15	16	14
Phe	24	29	21	21	28
Trp	2[i]		3	7	6
[Total]	[469]	[422]	[440]	[414]	[405]

[a] Analyses were determined at 24, 48, and 72 hr. Amino acid levels are calculated for mol wt 51,000. [b] Deuel *et al.* (1970). [c] Ginsburg (1972). [d] Ronzio *et al.* (1969). [e] Tate and Meister (1973). [f] Extrapolated to zero time. [g] Determined as cysteic acid, according to Moore (1963). [h] Highest value. [i] Estimated by the methods of Goodwin and Morton (1946) and Edelhoch (1967).

TABLE IV: Metal Ion Activation and Antagonism Toward Mn^{2+} in *B. stearothermophilus* Glutamine Synthetase Catalyzed Reactions.[a]

M^{2+}	r (Å)	% Activation Assay: (B)[b]	(T)[b]	Antagonism (% Change) (B)[b]
Mg	0.65	61	81	+10
Zn	0.74	5	4	−81
Co	0.78	24	48	−22
Ni	0.78	2	28	−53
Mn	0.80	*100*	*100*	(0)[c]
Cd	0.97	95	101	−2
Ca	0.99	4	27	+3
Hg	1.10	2	5	−81
Ba	1.35	0	4	−68

[a] Reactions carried out at 55°, with M^{2+} = nucleotide = 12.5 mM. In the antagonism experiments, other metal ions (12.5 mM) were added to the assay reaction already containing 12.5 mM Mn–ATP. [b] B = biosynthetic assay, pH 6.0; *T* = transferase assay, pH 6.5. [c] Total $[Mn^{2+}]$ = 12.5 mM.

was electrophoresed (Weber and Osborn, 1969), and its migration compared to several proteins of known size. The linear plot of log *M* *vs.* mobility shows that the *B. stearothermophilus* subunit has mol wt $\simeq$ 51,500 ± 1000. This, taken with the dodecameric structures observed in Figure 2, would indicate a native enzyme of mol wt about 620,000 ± 10,000. Thus the thermophilic enzyme appears to be slightly larger than the mesophilic bacterial ones.

Amino Acid Content. The levels of amino acids analyzed in acid hydrolysates of purified enzyme are listed in Table III, and are compared to those for glutamine synthetases from other sources. Overall, the analysis for the *B. stearothermophilus* enzyme appears to resemble that for the *B. subtilis* protein more closely than those for *E. coli* or more highly evolved proteins. Levels of particular classes of residues will be compared in a following communication with regard to the thermostability of the protein. The total number of residues is greater for the thermophilic protein than for the mesophilic ones, consistent with the higher molecular weight for the *B. stearothermophilus* enzyme.

Ultraviolet Absorption Spectrum. Purified native enzyme at 1 mg/ml was observed using a Cary 14 recording spectrophotometer. The ratio of A_{280}:A_{260} was *ca.* 1.69, indicating a distinct absence of bound adenylyl or other uv-absorbing groups other than amino acid side-chain moieties. A solution of 1 mg/ml of purified enzyme (Lowry method) has an absorption of 0.45 at 280 nm.

Kinetics and Activation Properties. Enzyme activity was found to be stimulated by divalent metal ions, the most effective of which were Mn^{2+}, Mg^{2+}, Cd^{2+}, and Co^{2+}, in that order, as shown in Table IV. There is no apparent correlation between ionic radius and activation with these ions. Forward transferase activity is stimulated more strongly by a wider variety of divalent ions than is the biosynthetic activity. In a second set of experiments the ability of these ions to compete with Mn^{2+}-stimulated activity was tested (Table IV). This shows that most nonactivating ions, except Ca^{2+}, nonetheless appear to antagonistically inhibit Mn^{2+} activity.

Each of these ions induced different pH–activity profiles for the enzyme, as with glutamine synthetases from other sources (Monder, 1965; Deuel and Stadtman, 1970; Ginsburg, 1972). Biosynthetic activity was observed (Figure 3A) with Mn^{2+}, Mg^{2+}, Cd^{2+}, and Co^{2+}, equimolar with ATP, over the range of pH 5–8. Mn^{2+} shows the highest relative activation, with an optimum at pH 6.0. Mg^{2+} in excess over ATP stimulates activity further, especially below pH 6.5. This may reflect relatively weaker Mg–ATP complexation at lower pH. Activation as a function of metal ion levels at these pH optima was also studied (Figure 3B). Both Mn^{2+} and Cd^{2+} peak at [metal] = [ATP], then decrease, but Mg^{2+} does not show this behavior. Similar patterns were reported with Mn^{2+} for the *B. subtilis* (Deuel and Stadtman, 1970) and *Bacillus licheniformis* (Hubbard and Stadtman, 1967) enzymes. This peaking was also observable for the *B. stearothermophilus* enzyme upon varying [ATP] with constant $[Mn^{2+}]$. This tends to suggest that the catalytically active species is a metal–ATP–enzyme complex. It does not exclude the possibility of metal enzyme complexes, however.

The substrate specificity of the *B. stearothermophilus* enzyme was investigated in terms of relative activities of substrate structural analogs in the biosynthetic assay (Table V). Substrate K_m values for the thermophile (55°) are quite comparable to those reported for a variety of mesophilic glutamine synthetases (*cf.* references in Tate and Meister, 1973; Shapiro and Stadtman, 1970). Use of NH_2OH in place of NH_3, however, shifted the substrate K_m's to 2.0, 5.0, and 0.5 mM for ATP, L-Glu, and NH_2OH at pH 6.5 in the forward transferase assay. Differences in activity with NH_3 analogs may reflect both structural (steric) factors and electronic (pK_a) effects. Among the L-Glu analogs, D-Glu competes somewhat with L-

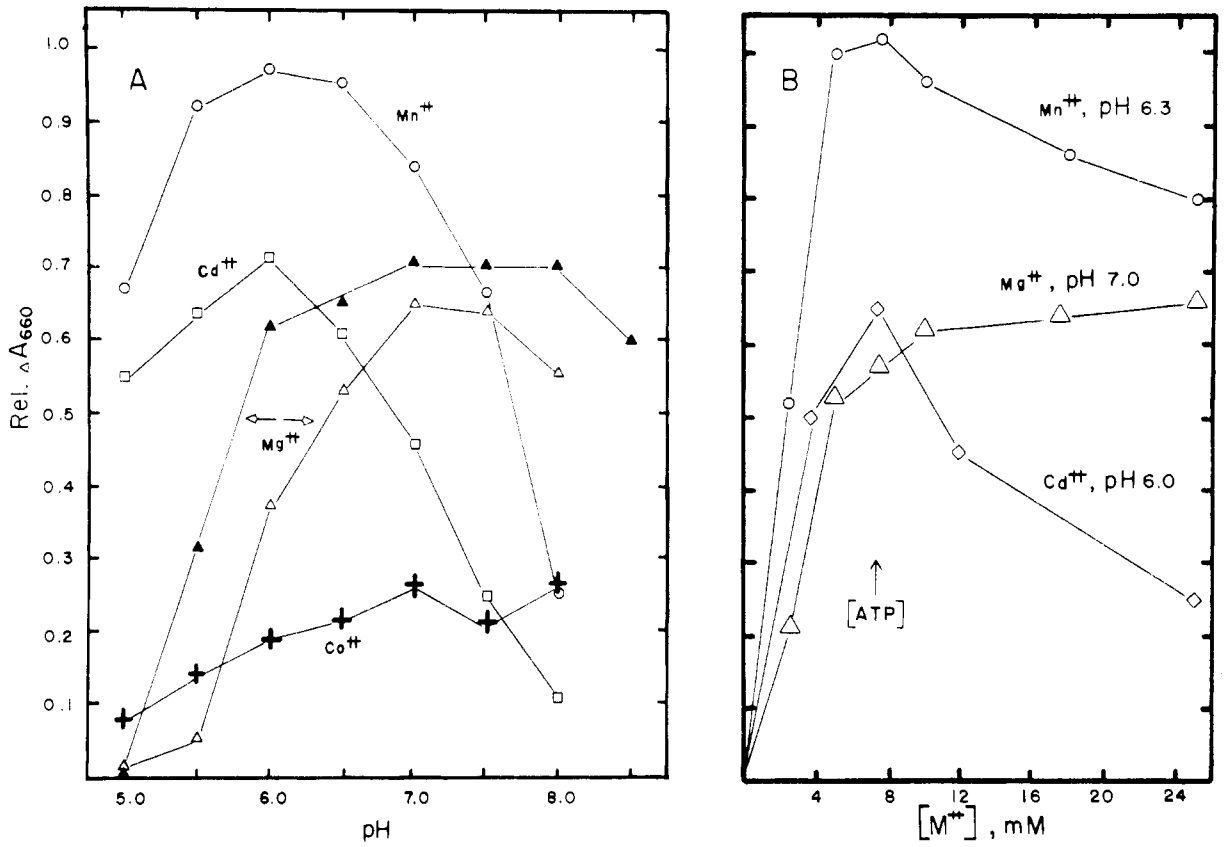

FIGURE 3: Activity profiles for *B. stearothermophilus* glutamine synthetase, using the biosynthetic assay (see Experimental Section). (A) Activation by various metal ions as a function of pH, at 55°, with $[M^{2+}] = [ATP] = 12.5$ mM. Mg^{2+} was used at 12.5 mM (open triangles) and 50 mM (filled triangles). (B) Effect of metal ion concentration relative to ATP (7.5 mM, arrow), at the indicated pH values, 55°.

Glu, but D-Glu and L-Asp are very poor substrates. Substitution of α-OH for α-NH_2 in L-Glu diminishes activity markedly, but methylation of α-NH_2 or substitution of methyl for the α-H causes less dramatic effects. Thus the enzyme may require a positively charged group at the α-NH_2 site, but lack strict steric requirements at the α-NH_2 or α-H loci. ATP and GTP are the best nucleotide triphosphate substrates by far.

Kinetics studies of the binding of substrates revealed that NH_3 and L-Glu bind with strictly hyperbolic kinetics, but that ATP shows cooperation binding, as shown in Figure 4. In this experiment, ATP and Mn^{2+} were maintained at equimolar levels throughout. Since the formation of the ternary E-Mn-ATP complex can give rise to nonhyperbolic kinetic responses (London and Steck, 1969) model simulation studies were carried

TABLE V: Specificity Studies of *B. stearothermophilus* Glutamine Synthetase.[a]

Substrate	Concn (mM)	Rel Act. (%)
NH_3 (K_m = 1.0 mM)	25	*100*
NH_2-CH_3	25	9.4
NH_2-OH	25	28
L-Glu (K_m = 1.5 mM)	10	*100*
D-Glu	10	10.5
L-Asp	10	2.1
D,L-Glu	10	81.5
L-α-Hydroxyglutarate	10	9.3
D-α-Hydroxyglutarate	10	2.4
D,L(α-CH_3)-Glu	10	34.3
D,L-(*N*-CH_3)-Glu	10	28.6
ATP ($S_{0.5} \simeq 0.30$ mM)	5	*100*
GTP	5	80.3
CTP	5	11.8
ITP	5	10.7
UTP	5	17.5

[a] Comparisons of activity induced by substrate analogs at fixed levels. The biosynthetic assay was used at pH 6.0, 55°, with Mn^{2+} = nucleotide.

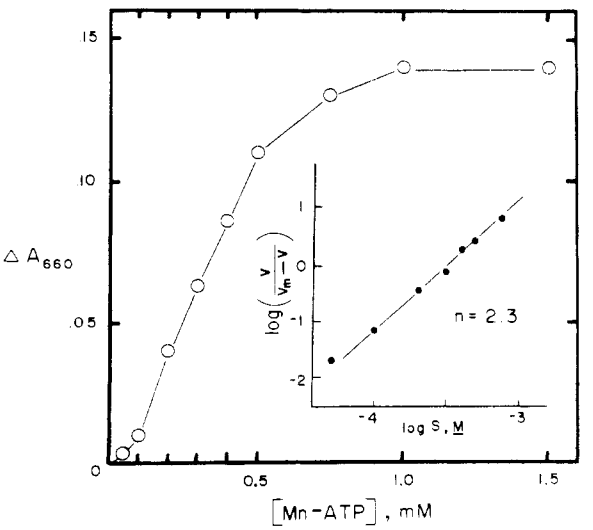

FIGURE 4: Activity of pure enzyme (biosynthetic assay) as a function of Mn-ATP concentration, pH 6.0, 55°, with other substrate levels held constant. Inset: Hill plot of log $(v/V_m - v)$ *vs.* log [ATP]. The calculated Hill $n = 2.3$.

out, assuming published values of pK_a's and association constants (Dawson *et al.*, 1969; Alberty, 1968). These calculations showed that the Mn-ATP complex is formed stoichiometrically at pH 6.0 at Mn^{2+} = ATP > 0.05 mM. Thus in the experiment of Figure 4, there is essentially no free Mn^{2+} or ATP, nor E-Mn or E-ATP. This implies that the sigmoidal velocity response is primarily attributable to enzyme binding Mn-ATP complex, not to multi-order kinetics or to competing equilibria. This also suggests, but does not prove, the existence of cooperative subunit interactions induced by Mn-ATP binding to the *B. stearothermophilus* enzyme.

To probe further the possibility of multiple conformations of the enzyme, enzyme biosynthetic activity was studied as a function of temperature. The Arrhenius plot in Figure 5 shows two distinct slopes, above and below 55°, with activation energies of 11.1 and 22.0 cal per mol, respectively. If preheating of the enzyme prior to assay was omitted, a monophasic plot was observed ($E_a \simeq$ 11.0 cal/mol; and 13.0 cal/mol for the transferase assay). During the 3-min preincubation and 15-min assay of Figure 5, little or no activity was lost until the temperature was 75° or above. By comparison, the *E. coli* ($E_{\overline{1.7}}$) and pea seed enzymes showed activity losses above 60 and 40° under similar assay conditions, respectively. The *E. coli* ($E_{\overline{1.7}}$), ovine brain, and pea seed enzymes exhibited linear and monophasic Arrhenius plots with E_a values of 15.7, 14.0, and 5.2 cal per mol, respectively.

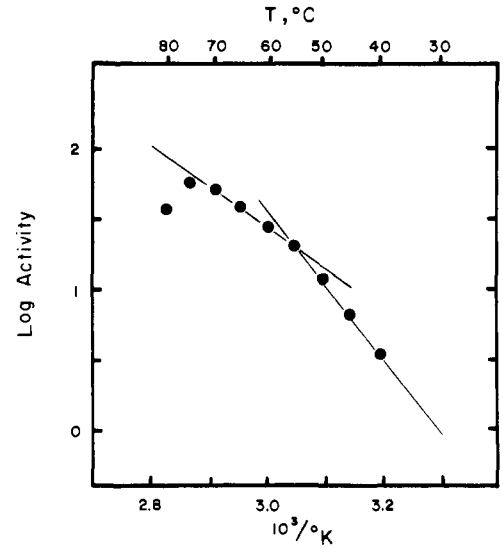

FIGURE 5: Arrhenius plot (log activity *vs.* $1/T$) for *B. stearothermophilus* glutamine synthetase, using the biosynthetic assay at pH 6.0. Enzyme was preincubated at each temperature for 3 min, then assay mixture was added and the assay carried out for 15 min. Activity was taken as ΔA_{660}, with correction for nonspecific ATPase activity.

Discussion

Synthesis of *B. stearothermophilus* glutamine synthetase at 55° is apparently controlled in the cell *via* a genetic repression mechanism that responds to ammonia or glutamine levels above ~2 mM. The carbon source appears to have little effect. Similar behavior has been reported for the *B. subtilis*, *B. licheniformis*, and *E. coli* systems (Deuel *et al.*, 1970; Hubbard and Stadtman, 1967; Woolfolk and Stadtman, 1967).

Development of methods for purification of this enzyme and the elucidation of its basic characteristics now allow for a more detailed inquiry into the complexities of its catalytic and regulatory mechanisms and the basis for its thermostability. The affinity gel chromatographic procedure reported here has allowed preparation of a completely homogeneous protein, and may prove useful in purifying enzymes from other sources.

Purification of this protein was unusually difficult, because the thermophilic proteins tended to precipitate similarly and were less readily fractionated by high salt, low pH, or combinations of these. This contrasts sharply to behavior in preparative procedures for the mesophilic enzymes and suggests that specific features of the surface of the thermophilic enzyme may differ from mesophilic ones. As with the *E. coli* enzyme, this protein is also somewhat labile on DEAE-cellulose, but stands up well in rather harsh 25° acetone treatment. Based on these preliminary characterization studies, this enzyme resembles but differs in subtle ways from other bacterial glutamine synthetases in terms of quaternary structure, subunit molecular weight, and amino acid composition. The maximal assay temperature is 70°, fully 15° higher than for the mesophilic bacterial enzymes under similar conditions. Specificity for the natural substrates (relative to analogs) is quite high at 55°, and substrate K_m values are quite comparable to those reported for mesophilic enzymes, as are the catalytic activation energies.

The kinetic behavior of the thermophilic enzyme appears less complex than with other glutamine synthetases, *e.g.*, there is a distinct lack of substrate inhibition or activation by NH_3 or glutamate (Deuel and Stadtman, 1970; Hubbard and Stadtman, 1967). The cooperativity of Mn-ATP binding represents a rather novel and intriguing observation for glutamine synthetases. Cooperative substrate binding is not an uncommon property of thermophilic enzymes in general (*cf.* review by Singleton and Amelunxen, 1973), nor are nonlinear Arrhenius plots.

The *E. coli* enzyme has been reported to show sigmoidal responses to L-glutamate, induced by Co^{2+} but not other ions (Segal and Stadtman, 1972). Deuel and Stadtman (1970) reported nonhyperbolic responses of the *B. subtilis* enzyme to variation of $MnCl_2$ with constant ATP levels, and also non-Michaelis-Menten saturation by Mn-ADP in a reverse transferase assay (Deuel and Turner, 1972). There were also marked substrate activations and inhibitions by L-glutamate and ammonia (Deuel and Stadtman, 1970), dependent on the metal ion present, but the *B. stearothermophilus* enzyme fails to exhibit any such effects under any conditions studied so far.

The nonlinear Arrhenius plot produced only by preincubation of enzyme may suggest slow conformational changes or hysteretic effects in the protein (Bates and Frieden, 1973a,b), most likely related to ATP binding. *In vivo* these effects could play both a regulatory and a protective role. Whether other *Bacillus* glutamine synthetases show cooperative ATP binding is not clearly established (Deuel and Turner, 1972).

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