Glutamine Synthetase of *Bacillus stearothermophilus*. II. Regulation and Thermostability†

Frederick C. Wedler* and F. Michael Hoffmann

ABSTRACT: Feedback regulation of glutamine synthetase from *Bacillus stearothermophilus* is observed with AMP, ADP, GDP, CTP, glycine, L-alanine, L-histidine, L-tryptophan, carbamyl phosphate, and glucosamine 6-phosphate. AMP and ADP are potent competitive inhibitors of ATP, and their inhibition correlates with adenylate energy charge values of 0.75–0.95. As deduced from interactions of substrate pairs and from competitive inhibition studies, the thermophilic enzyme possesses fewer separate inhibitor binding sites than its mesophilic counterparts. CTP appears to compete directly with L-glutamate, but L-histidine and glucosamine-6-P compete partially with both ATP and L-glutamate. Glycine inhibits V_{max}, whereas the effects of GDP and L-alanine depend partially on levels of bound substrates and inhibit both V_{max} and the K_m of L-glutamate. L-Gln inhibits only very weakly. In contrast to other *Bacillus* sp. enzymes, little or no synergism of inhibition occurs with modifier pairs. Thermostabilization of the protein appears to be provided by a combination of effects. Ligands interacting at the glutamate binding site markedly thermostabilize the enzyme. Among the divalent ions tested, weak stabilization occurs with $Hg^{2+} > Cd^{2+} > Mn^{2+}$, and others have no effect. In comparison to mesophilic enzymes, thermostability correlates with both increased average hydrophobicity and with carboxylate side-chain residue content, but not with α-helix, β-sheet, or hydrogen-bonding residues. Aggregation of native dodecamers is driven by heat, is dependent on protein concentration, salt, and pH, and strongly protects against thermal denaturation. Such aggregation, it is proposed, could provide both a regulatory mechanism and a survival advantage to key enzymes during evolution and in maintenance of life in extreme environments.

Because glutamine is a key precursor for a multiplicity of biosynthetic pathways, the activity of glutamine synthetase is closely controlled. As reviewed by Shapiro and Stadtman (1970), with emphasis on the *Escherichia coli* system, these regulatory mechanisms can include repression by glutamine and ammonia, inhibition by high levels of substrates or products, response to different divalent metal ions, the action of nitrogeneous feedback modifiers derived from glutamine, and covalent derivatization (adenylylation) of the enzyme. Depending on the source, a given enzyme may exhibit these control properties to varying degrees. As seen in the accompanying paper (Wedler and Hoffmann, 1974), the thermophilic *Bacillus stearothermophilus* enzyme differs from mesophilic *Bacillus* sp. enzymes in lacking substrate inhibition or activation phenomena. Results from the present research indicate other basic changes and a minimization in regulatory binding sites and their interactions, especially the synergistic effects observed for *Bacillus subtilis* or *Neurospora crassa* (Hubbard and Stadtman, 1967; Deuel and Stadtman, 1970; Deuel and Prusiner, 1974).

Temperature has a profound effect on macromolecular evolution (Brock, 1967, 1970). It is intriguing to consider the complexity of function maintained by cells in extreme thermal environments and how this is accomplished, especially in key regulatory enzymes such as glutamine synthetase.

Although there are numerous current theories of effects considered crucial for protein thermostability (*cf.* reviews by Amelunxen and Lins, 1969, Howell *et al.*, 1969, and Singleton and Amelunxen, 1973), no single one is observed to correlate universally. This research indicates that a combination of effects apparently is utilized to thermostabilize *B. stearothermophilus* glutamine synthetase.

† From the Chemistry Department, Cogswell Laboratory Rensselaer Polytechnic Institute, Troy, New York 12181. *Received March 20, 1974.* This research was supported in part by the Petroleum Research Fund, administered by the American Chemical Society, and from the National Science Foundation (Grants GB34751 and GU3182).

Experimental Section

Materials. Bacillus stearothermophilus was grown on limiting ammonia–dextrose media and glutamine synthetase was purified to homogeneity as described previously (Wedler and Hoffmann, 1974). All biochemical reagents were of highest purity available from Sigma Chemical Co. All inorganic compounds were of ACS analytical grade supplied by Fisher Scientific. All water was double distilled from glass.

Methods. Enzyme activity was assayed by the standard biosynthetic (P_i release) or forward transferase (γ-glutamylhydroxamate formation) assays described previously (Wedler and Hoffmann, 1974). In addition to the appropriate control reactions, these procedures involved preheating the enzyme at 55° with assay mixture lacking ATP for 2–3 min prior to initiation of the reaction by addition of ATP. This procedure minimizes ATP hydrolysis and thus the levels of AMP and ADP present, which could give very nonlinear kinetics. Optical density changes, corresponding to <0.5 mM product formed, were found to give linear kinetics in both assays with saturating substrates, but levels >0.1 mM ADP product were not exceeded with limiting ATP.

Circular dichroism measurements were carried out with a Jasco Model ORD/UV-5 equipped with Sproul Scientific 55-15 CD modification. A 1.0-mm stress-free quartz cell was used and the instrument calibrated with *d*-10-camphorsulfonic acid (Aldrich) and with α-chymotrypsin (Worthington). Protein concentrations of about 0.025% were used for all measurements. A computer program kindly supplied by Dr. J. T. Yang, and a least-squares program BMDX85 from the UCLA Health Sciences Computing Facility, were used for data analysis on an IBM 360/50 computer. Some recomputation of Yang's standard curves was also carried out for analysis of our protein data.

Results

Regulatory Properties. Of the various metabolic products and intermediates tested as potential inhibitors of *B. stearoth-*

TABLE I: Effects of Feedback Modifiers on *Bacillus stearothermophilus* Glutamine Synthetase.[a]

Assay	Modifier	mM	% Inhibition at Substrate Levels: Sat.	Lim. NH_3 or NH_2OH	Lim. Glu	Lim. ATP–M^{2+}
B	None		0	0	0	0
	Gly	10	50[2]	55[20]	62[8]	55[3]
	L-Ala	10	58[*st.*][c]	55[19]	62[5]	43[5]
	L-His	10	16[10]	11[18]	25[*st.*]	33[24]
	L-Trp	6.2	9[*st.*]	0[6]	0[*st.*]	0[*st.*]
	GN-6-P[d]	10	50[56]	37[43]	37[68]	50[68]
	AMP	10	90[90]	88[83]	90[87]	99[83]
	ADP	5	55[60]	38[61]	62[*st.*]	99[10]
	GDP	10	18[45]	25[42]	37[10]	17[20]
	CTP	10	20[38]	21[36]	75[52]	20[41]
	C-P[d]	10[b]				
T	None		0	0	0	0
	Gly	25	12	18	*st.*[c]	*st.*[c]
	L-Ala	25	13	~2	*st.*	~2
	L-His	25	10	6	*st.*	5
	L-Trp	12.5	*st.*[c]	0	*st.*	*st.*
	GN-6-P[d]	12.5	*st.*	84	*st.*	*st.*
	AMP	25	83	50	77	84
	ADP	12.5	81	78	82	91
	GDP	25	*st.*	*st.*	*st.*	11
	CTP	12.5	*st.*	*st.*	20	12
	C-P[d]	5	*st.*	23	23	8

[a] Using the biosynthetic (B) and forward transferase (T) assays, 55° (see Experimental Section). With limiting conditions NH_3, Glu, and M^{2+}–ATP were reduced to 2.0, 2.0 and 0.5 mM, respectively, in the biosynthetic assay; NH_2OH, L-Glu, and ATP were reduced to 0.5, 5.0, and 1.0 mM, respectively, in the transferase assay. Inhibitions with the Mn^{2+}-stimulated assays were observed at pH 6.0 with Mn^{2+} = ATP; those with $MgCl_2$ were with 25 mM $MgCl_2$ at pH 7.0 [brackets]. [b] Too labile to assay by P_i release at 55°. [c] Stimulates activity. [d] GN-6-P = glucosamine-6-phosphate; C-P = carbamyl phosphate.

ermophilus glutamine synthetase, only those listed in Table I caused a >5% change in activity. No inhibitions were observed with IMP, NAD^+, $NADP^+$, nicotinic acid, or anthranylic acid, as observed with *N. crassa* (Kapoor and Bray, 1968). L-Glutamine exerts a much weaker inhibitory effect than with other systems (Ravel *et al.*, 1965; Deuel and Stadtman, 1970; Hubbard and Stadtman, 1967). There are marked differences in the effects induced by these modifiers that depend upon whether Mn^{2+} or Mg^{2+} are present and whether NH_3 or NH_2OH are used in the assay system, as well as whether substrate levels are limiting or saturating. In the Mn^{2+} biosynthetic assay, L-Trp and glucosamine-6-P appear to require near-saturating substrate levels to exert their effect. With the Mg^{2+} biosynthetic assay, the inhibitions observed (relative to Mn^{2+} activity) are weaker for glycine, L-Ala, L-His, and L-Trp, but somewhat stronger for glucosamine-6-P, GDP, and CTP. Overall, with Mn^{2+} present, transferase activity is inhibited less strongly than biosynthetic activity. The basis for the stimulation of activity by several of these end products is not understood at this point. For the phosphate-containing molecules, release of P_i or a P_i contamination could allow for reverse transferase activity and may explain these results in part.

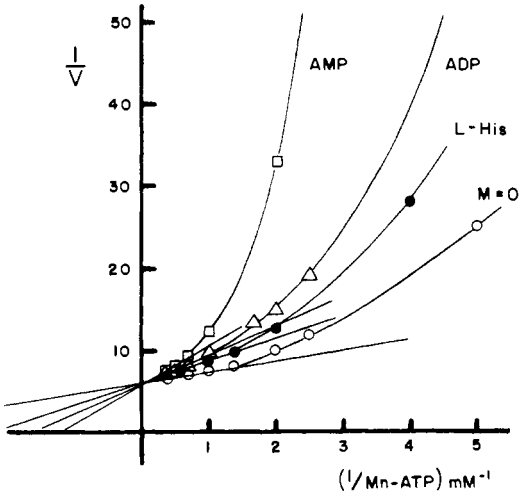

FIGURE 1: Effect of feedback modifiers on *B. stearothermophilus* glutamine synthetase, upon variation of *Mn-ATP concentration* in the biosynthetic assay, 55°, pH 6.0 (see Experimental Section for details) as illustrated by Lineweaver–Burk reciprocal plots. Assays contained (in mM) 40 L-Glu, 40 NH_4Cl, 20 imidazole, 100 KCl with 0.25 mM ADP (triangles), 0.25 mM AMP (squares), or 10 mM L-His (closed circles), added. Mn^{2+} levels were kept equal to total nucleotide.

The Mn^{2+}-stimulated biosynthetic assay also was used to study how these modifiers compete with substrates. ADP and AMP exert the strongest effects with limiting ATP (Table I) and thus may compete directly with ATP, as may L-His also. These possibilities were probed by the experiments illustrated in Figure 1, in which Mn-ADP, Mn-AMP, and L-His are seen to alter the Mn-AMP binding ($S_{0.5}$ value) but not the V_{max} value. The nonhyperbolic nature of the Mn-ATP binding is also illustrated in those double-reciprocal plots.

These observations suggest that adenylate energy charge (Atkinson, 1968) may provide an important regulatory mechanism for this enzyme, and so the data of Figure 1 were thus replotted as relative rate (V_{obsd}/V_{max}) *vs.* energy charge, shown in Figure 2, calculated separately for ADP and AMP. These

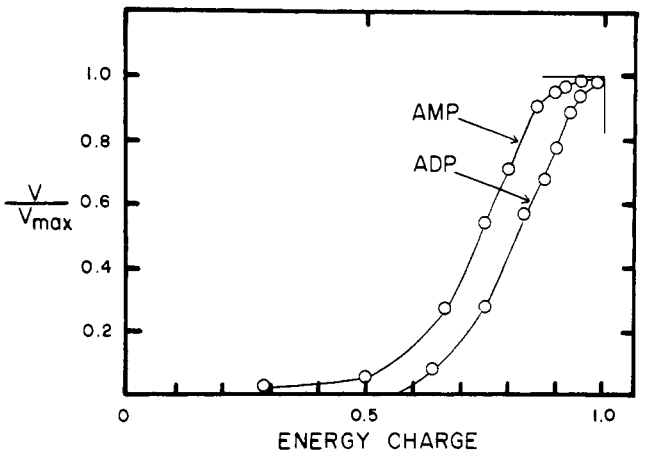

FIGURE 2: Effect of adenylate energy charge on *B. stearothermophilus* glutamine synthetase activity, calculated from the data of Figure 1. Energy charge is defined as ([ATP] + (½)[ADP])/([ATP] + [ADP] + [AMP]), and was calculated separately for inhibitions by AMP and ADP, as shown.

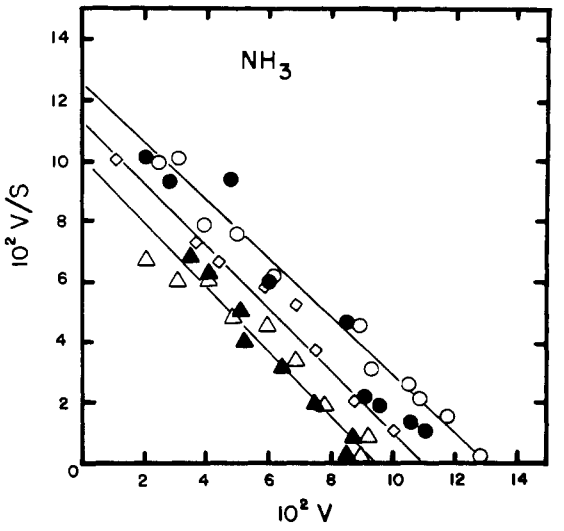

FIGURE 3: Effect of feedback modifiers on *B. stearothermophilus* glutamine synthetase, upon variation of *ammonia concentration* in the biosynthetic assay, 55°, pH 6.0. Substrate levels were as in Figure 1, except that Mn-ATP was 2 mM. Velocity data are presented in an Eadie-Hofstee plot. Symbols: no modifier (open circles), 2.5 mM glucosamine-6-P (closed circles), 1.0 mM CTP-Mn (open triangles), 2.5 mM glycine (closed triangles), and 0.25 mM Mn-AMP (diamonds).

plots show that a marked variation in rate occurs in the region of energy charge values of 0.75–0.95, as would be expected for a biosynthetic regulatory enzyme.

Several of the more potent modifiers were tested as competitors with respect to ammonia, namely, glycine, glucosamine-6-P, AMP, and CTP. In the Eadie-Hofstee plot of Figure 3 each of these is seen to alter the V_{max} (x intercept) but not the K_m (slope), that is, each is purely noncompetitive relative to ammonia. The effect of glucosamine-6-P was weak and difficult to interpret.

Based on effects observed in Table I, a wider variety of modifiers was tested as competitors of L-glutamate in Figure 4. Both CTP and glucosamine-6-P appear to compete directly with L-Glu, whereas AMP is noncompetitive. Glycine is also noncompetitive, but L-alanine and GDP give mixed inhibitions, altering both V_{max} and K_m.

In order to interpret the data of Figures 1, 3, and 4 in terms of the number of separate modifier sites, the effect of selected pairs of substrates was observed, as presented in Table II. From the level of inhibition observed with each modifier alone, calculations were made to predict per cent inhibition with both modifiers present, depending upon whether their effects were additive, cumulative, or antagonistic (Woolfolk and Stadtman, 1967). The action of glycine plus other inhibitors generally indicates cumulative or additive inhibition, as with Gly paired with CTP, AMP, L-Ala, and L-His, but it appears that Gly plus L-Gln act antagonistically. Alanine acts cumulatively with CTP or L-His but antagonistically with AMP or L-Gln. L-His

TABLE II: Inhibition by Modifier Pairs with *Bacillus stearothermophilus* Glutamine Synthetase.[a]

Modifier:	Observed			Calculated		
	A [%*i*]	B [%*i*]	A + B [%*i*]	Additive	Antagonistic	Cumulative[b]
	Gly[16]	CTP[24]	37	40	<24	36
	Gly[16]	AMP[66]	89	100	<66	71
	Gly[16]	L-Gln[5]	6	31	<16	20
	Gly[16]	L-His[10]	26	26	<16	24.4
	Gly[16]	L-Ala[28]	37	44	<28	23.5
	L-Ala[28]	CTP[24]	47	52	<28	45
	L-Ala[28]	AMP[66]	49	94	<66	75
	L-Ala[28]	L-Gln[5]	3	33	<28	32
	L-Ala[28]	L-His[10]	35	38	<28	35
	L-His[10]	CTP[24]	37	34	<24	31
	L-His[10]	AMP[66]	57	76	<66	69
	L-His[10]	L-Gln[5]	22	15	<10	14
	L-Gln[5]	CTP[24]	20	29	<24	28
	L-Gln[5]	AMP[66]	77	71	<66	68
	AMP[66]	CTP[44]	81	100	<66	81

[a] The standard Mn^{2+}-stimulated biosynthetic assay was used, pH 6.5, 55°. Substrate levels were (in mM): NH_2OH, 2.0; L-Glu, 10.0; and MnATP, 5.0. The inhibition with each modifier alone is shown in brackets. The calculated values are for model inhibitions where modifiers interact in an additive manner, at a single site, or cumulatively (see text). [b] Calculated according to the formula $X + (Y/100)(100 - X)$, where X and Y are the values of per cent inhibition observed with modifiers A and B alone, respectively.

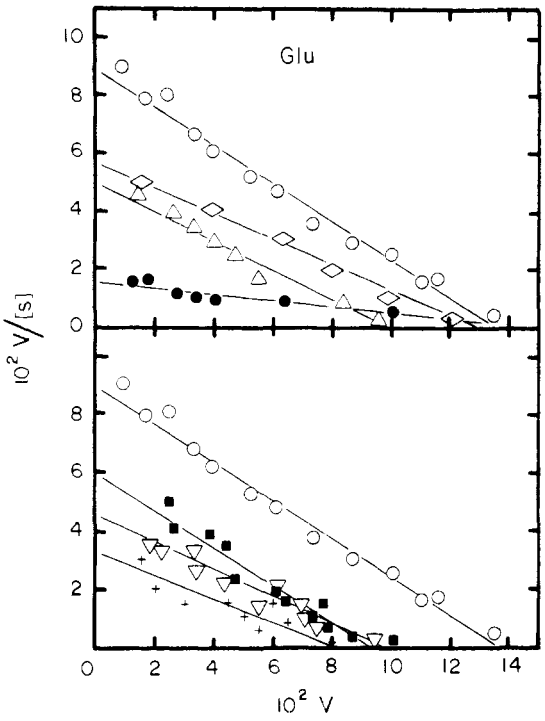

FIGURE 4: Effect of feedback modifiers on *B. stearothermophilus* glutamine synthetase, upon variation of L-*glutamate concentration*, in the biosynthetic assay, 55°, pH 6.0. Substrate levels were as in Figure 1, except that Mn–ATP was 2 mM. Velocity data are shown as an Eadie–Hofstee plot of *v/s vs. v*. Symbols: no modifier (open circles), 2.0 mM CTP–Mn (closed circles), 2.5 mM glucosamine-6-P (diamonds), 0.25 mM Mn–AMP (upward triangles), 2.5 mM L-Ala (downward triangles), 2.5 mM Gly (closed squares), and 2.5 mM GDP (crosses).

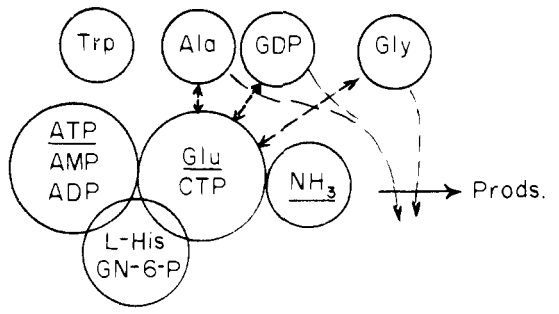

FIGURE 5: Modifier binding domains relative to substrate binding sites (underlined) on the surface of *B. stearothermophilus* glutamine synthetase, as deduced from inhibition data (see text) for Mn^{2+}-stimulated biosynthetic activity, pH 6.0. Direct competitions are indicated by inclusion of these ligands within a given domain, and modifiers competing for more than one domain are shown in intersecting circles. Those modifiers interacting with a domain, dependent upon bound substrate or inhibiting catalysis from a separate site are indicated by broken arrows.

acts in an additive manner with CTP, antagonistically with AMP, but shows slight synergism with L-Gln. L-Gln and CTP are antagonistic, but the effects of L-Gln and AMP are additive. AMP and CTP effects are cumulative.

The data of Tables I and II and Figures 1–4 may be interpreted as to the minimal binding domains and interactions on the surface of the enzyme, for the Mn^{2+}-stimulated biosynthetic assay, as shown in Figure 5. AMP and ADP compete for the ATP site. L-Gln and CTP compete for the L-Glu site. L-His and glucosamine-6-P appear to compete weakly for both the ATP and L-Glu sites. Glycine appears to alter V_{max} but also to interact with the L-Glu site, as to L-Ala and GDP, especially at limiting levels of L-Glu. The action of L-Trp is weak and as yet undetermined, but apparently depends on bound substrates. This scheme can be used as a working model for more extensive studies involving variation of substrate and modifier concentrations.

This representation does not account for such aspects of the regulation as the influence of different divalent metal ions or stimulatory effects. The slight synergism noted between the modifiers L-His and L-Gln (Table II) may result from homotropic or heterotropic subunit interactions induced at the ATP site by L-His, although AMP and CTP do not show this synergism. The number of separate modifier binding sites in this model is somewhat fewer than that suggested by data for mesophilic *Bacillus* sp. enzymes. The complexity of regulatory site interactions, *e.g.*, synergism, is also diminished considerably (Hubbard and Stadtman, 1967; Deuel and Stadtman, 1970; Deuel and Prusiner, 1974).

Thermostabilization. To probe whether rapid degradation and resynthesis contributes to survival of the enzyme at 55°, the following experiment was conducted. *B. stearothermophilus* cells were grown to mid-late-log phase in defined media with limiting NH_4Cl (1 mM), then rapidly divided into two portions. The first portion was harvested, and the second was incubated 2 hr at 55° after immediate addition of 5 mM NH_4Cl to repress enzyme resynthesis, then harvested. After breaking the cells and purifying the enzyme through step 1 of the standard procedure (Wedler and Hoffmann, 1974), the specific activities were compared and found to be identical. This also further emphasizes that the enzyme is inherently thermostable *in vivo*.

A variety of ligands were examined for their ability to enhance or prevent thermal denaturation of the enzyme at 65° (Table III). Among the divalent metal ions tested, Ni^{2+} enhanced denaturation while Mg^{2+}, Zn^{2+}, and Co^{2+} had essentially no effect. Thermostabilization was afforded by $Hg^{2+} > Cd^{2+} > Mn^{2+}$. There is no obvious correlation between thermostabilization and stimulation of activity, ionic radius, or type of metal ion among these. Those metal ions which best activate the enzyme are generally the poorest thermostabilizing agents. Thus, these effects appear to be nonspecific and may not involve association in the active-site region.

Among the substrates tested, ammonia lends no stability, ATP only a slight effect, but glutamate provides very strong stabilization. The effect of any single ligand is enhanced by pairing: either NH_3 and L-Glu, NH_3 + ATP, or L-Glu + ATP. Modifiers that interact with the glutamate site (see above section) give the strongest thermostabilization, namely, L-Ala, CTP, and L-Gln. Glycine and L-His also stabilize significantly, but carbamyl-P, glucosamine-6-P, and L-Trp do not. ADP and AMP stabilize about as well as does ATP. These observations are consistent with a significant substrate protection mechanism at the glutamate site for this thermophilic enzyme, and also support the binding site scheme of Figure 5.

Next, the intrinsic properties of the protein were examined for factors that correlate with thermostability. The amino acid analyses for several glutamine synthetases presented in the accompanying paper (Table III, Wedler and Hoffman, 1974) were examined in terms of (a) the average hydrophobicity of the proteins (Bigelow, 1967; Tanford, 1962), (b) the number of residues capable of hydrogen bonding, and (c) the total number of aspartate and glutamate residues present. There was essentially no trend with regard to H-bonding residues, but a positive correlation (about 10%) with regard to average hydrophobicity ($H\phi$) and Asp + Glu content, as measured by the half-time for denaturation at 70°.

TABLE III: Protection against Heat Denaturation of *Bacillus stearothermophilus* Glutamine Synthetase by Metal Ions, Substrates, and Modifiers.[a]

Adduct	Concn (mM)	% Act. Loss
0		78
Mg^{2+}	10	77
Zn^{2+}	10	81
Co^{2+}	10	81
Ni^{2+}	10	>90
Mn^{2+}	10	69
Cd^{2+}	10	43
Ca^{2+}	10	79
Hg^{2+}	10	25
NH_3	50	77
L-Glu	50	6.1
ATP	10	66
NH_3 + L-Glu	50 and 50	0
NH_3 + ATP	50 and 10	30
L-Glu + ATP	50 and 10	0
Gly	12.5	13
L-Ala	12.5	5
L-His	12.5	24
L-Gln	12.5	0
Carbamyl-P	12.5	71
Glucosamine-6-P	12.5	70
AMP	12.5	66
ADP	12.5	64
GDP	12.5	56
CTP	12.5	21
L-Trp	6.2	77

[a] A sample of diluted enzyme (0.1 mg/ml) was first assayed with added ligand at 55° (biosynthetic reaction), then incubated at 65° for 20 min, then rapidly cooled to 25°, then reassayed at 55°.

Preliminary circular dichroism measurements on both the *B. stearothermophilus* and *E. coli* glutamine synthetases at 25° in pH 7.0 buffer, ionic strength 0.1 M, indicate about 42% less α-helical structure but about 21% *more* β-sheet structure in the thermophilic protein than in the mesophilic one (Hunt and Ginsburg, 1972). Using the α-helix and β-sheet formation factors, P_α and P_β, recently derived by Chou and Fasman (1974), there is no trend noted between the *B. stearothermophilus and E. coli* proteins, using the formulas

$$\alpha = \Sigma n_i P_{\alpha i} / \Sigma n_i$$

and

$$\beta = \Sigma n_i P_{\beta i} / \Sigma n_i$$

where n_i is the number of residues of a given type per subunit, and the terms α and β are the relative probability of α-helix or β-sheet structures forming, ignoring sequence considerations.

These data, taken together, may thus suggest subtle but critical rearrangements of different types of amino acid residues in the thermophilic enzyme that lend heat stability.

In the course of purifying the enzyme (Wedler and Hoffmann, 1974), it was observed that the dependence of activity on protein concentration was nonlinear. Figure 6A shows several such plots at different specific activities of protein. These are typical of effects observed with a change in protein solubility due to aggregation (Dixon and Webb, 1964). Since the change in slope occurs at lower and lower concentrations of protein as the specific activity increases, this suggests aggregation of the enzyme with itself rather than with other proteins.

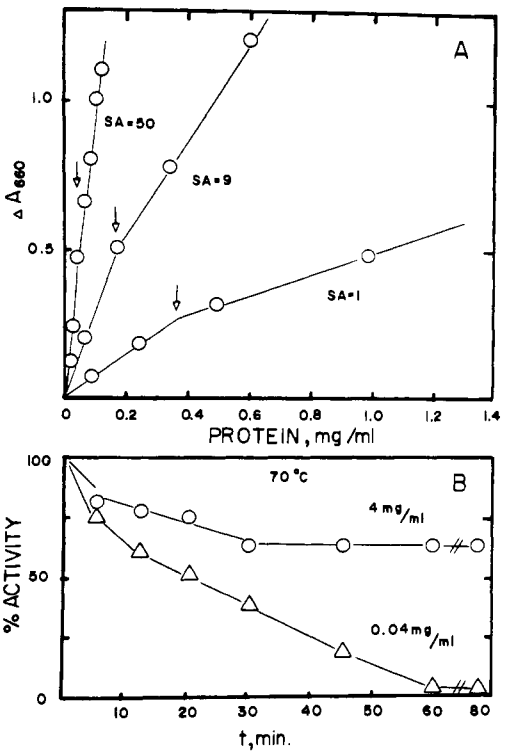

FIGURE 6: Effect of protein concentration on *B. stearothermophilus* glutamine synthetase activity. (A) During purification, using the forward transferase assay with $MnCl_2$, 55°. (B) During heat denaturation at 70° as a function of time, using purified protein at 4 mg/ml (circles) and 0.04 mg/ml (triangles).

Enzyme concentration is also observed to have a distinct effect on thermostability, as seen in Figure 6B. At 0.04 mg/ml of protein at 70° the activity is lost completely with a half-time of 20–25 min, but at 4.0 mg/ml of protein the activity is only partially lost, then remains stable.

To interpret these observations, one must consider evidence for all possible states of aggregation and for other effects. Unlike the *B. subtilis* enzyme (Deuel and Stadtman, 1970) native dodecamer does not dissociate to subunits upon dilution in the absence of metal ions, since species of mol wt <600,000 were not observed on Agarose 0.5m or in electron micrographs. However, with concentrated enzyme, we have observed aggregates of the dodecamer as excluded peaks on Agarose 1.5m (Wedler and Hoffman, 1973). Even dilute enzyme solutions show some clustering of dodecamers on electron micrographs (Wedler and Hoffman, 1974). During the development of the purification scheme for the enzyme, it was observed that the protein could be reversibly precipitated at pH 5.6 upon treatment with 20% saturated ammonium sulfate at 70°. Thus, it is proposed that the data of Figure 6 indicate that very dilute protein does not aggregate and is readily dissociated and denatured by heat (but not upon dilution alone), and that concentrated enzyme can aggregate to give species which are more thermostable then native dodecamer, for reasons which will be discussed. The nature and degree of this aggregation process remains as an intriguing topic for future experimentation.

Discussion

The glutamine synthetase of *B. stearothermophilus* is distinctly different from its mesophilic counterparts in several regards. High levels of substrates produce no inhibitions or acti-

vations, but the kinetically observed binding of ATP shows homotropic cooperativity (Wedler and Hoffman, 1974). The spectrum of modifiers inhibiting the enzyme is as wide, but the number of separate modifier sites would appear to be fewer in the thermophilic case, and synergistic inhibitory interactions between AMP and L-His or L-Gln are absent. Although the enzyme is distinctly more thermostable, it retains most of the basic functional information present in mesophiles, but it appears to accomplish this in a more direct, simplified manner.

Current hypotheses for mechanisms of enzyme thermophily involve both intrinsic and extrinsic effects. The *intrinsic* factors may include (a) altered amounts of secondary structure and by implication the levels of H-bonding residues, as with thermophilic enolase (Barnes and Stellwagen, 1973) or (b) increased apolar interactions (Brandts, 1967; Bigelow, 1967; Goldsack, 1970), and (c) increased disulfide-bond cross-linking. Among the *extrinsic* factors which may thermostabilize, one must include (a) metal ions, as with Ca^{2+} and thermolysin (Feder *et al.*, 1971; Colman *et al.*, 1972) or Mg^{2+} with yeast enolase (Brewer and Weber, 1966), and (b) bound substrates of cofactors, as reviewed by Singleton and Amelunxen (1973). Substrate protection occurs, for example, with Arg-tRNA synthetase from *B. stearothermophilus* (Parfait, 1973). The present study indicates that several of these factors are utilized to thermostabilize *B. stearothermophilus* glutamine synthetase: protection by ligands bound to the L-glutamate site, altered levels of β-sheet and α-helical structures, increased levels of carboxylate *and* apolar side-chain residues, slight protection by metal ions, and aggregation of dodecamers. *In vivo* no single effect is likely to suffice; rather, a combination must be used. Association of glutamate or of metal ions may involve stabilizing a single defined region, whereas the other effects may protect more extensive portions of the protein core or surface. Since thermostability correlates with increases in both apolar and polar residue contents, rearrangement or clustering of similar residues on the protein surface seems likely. Since aggregation is favored by high salt and heat, this suggests apolar "patches" on the upper and lower surfaces of the stacked hexagons of the dodecameric native enzyme. Increased β-sheet content may relate to stronger intersubunit bonding such as that found in lactate dehydrogenase (Rossmann *et al.*, 1973) either within or between hexamers in dodecamers or in formation of oligomers.

Aggregation should be considered at two distinct stages: first, formation of native dodecamer, then formation of higher oligomers. In the primative environment the first step is an obvious evolutionary precursor to the evolution of more sophisticated fine tuning of enzymes by homotropic and allosteric subunit interactions. According to the "hydrophobic" theory of apolar interactions (Eisenberg and Kauzmann, 1969), elevated temperatures should favor the aggregation of apolar peptide chains by releasing water of solvation and thus increasing entropy. Once aggregated, the protein surface area exposed to denaturing solvent would be reduced. A comparison of molecular weights and subunit structures for mesophilic and thermophilic enzymes (Singleton and Amelunxen, 1973) indicates that thermophilic proteins are comparable in size and subunit structure to mesophilic ones, but in some cases are diminished in functional complexity. The ability of glutamine synthetase to form oligomers or high aggregates is a property apparently present in several bacterial enzymes, mesophilic as well as thermophilic (Valentine *et al.*, 1968).

Stellwagen *et al.* (1973) observed aggregation of thermophilic enolase at high temperature but did not regard this as functionally important. It seems reasonable to propose that protein aggregation could be used *in vivo* for several purposes. The first is thermostabilization, supported by the thermal denaturation curves in Figure 6B. The second is regulation, suggested by the nonlinear concentration-activity plots in Figure 6A. Although these plots can be altered by temperature, pH, ionic strength, and bound ligands, the contribution of each of these parameters is poorly defined at present. Whether aggregation is a general property of thermophilic enzymes also awaits further study. Finally, the aggregation phenomenon raises the question whether the nonlinear Arrhenius plot in the previous paper (Wedler and Hoffmann, 1974) results from an enzyme conformational change, *e.g.*, that associated with ATP binding, or from a temperature-dependent change in aggregation. Because enzyme levels used in the assays were far below the "break" points in the concentration-activity plots, it seems likely that a conformational change is the more likely explanation.

Those phenomena which deserve particular attention in future studies include the parameters which control aggregation, the mechanistic basis for each specific thermostabilization effect, and further studies on the mechanisms of substrate binding, catalysis, and regulation.

Acknowledgments

Mrs. JoAnn Cencula is thanked for her technical help and Mr. Greg McClune for carrying out the circular dichroism experiments.

References

Amelunxen, R. E., and Lins, M. (1969), *Arch. Biochem. Biophys. 125*, 765.
Atkinson, D. E. (1968), *Biochemistry 7*, 4030.
Barnes, L. D., and Stellwagen, E. (1973), *Biochemistry 12*, 1559.
Bigelow, C. C. (1967), *J. Theor. Biol. 16*, 187.
Brandts, J. F. (1967), *in* Thermobiology, Rose, A. H., Ed., New York, N. Y., Academic Press, p 25.
Brewer, J. M., and Weber, G. (1966), *J. Biol. Chem. 241*, 2550.
Brock, T. D. (1967), *Science 158*, 1012.
Brock, T. D. (1970), *Annu. Rev. Ecol. Syst. 1*, 191.
Chou, P. Y., and Fasman, G. D. (1974), *Biochemistry 13*, 222.
Colman, P. M., Jansonius, N. N., and Matthews, B. W. (1972), *J. Mol. Biol. 70*, 701.
Deuel, T. F., and Prusiner, S. (1974), *J. Biol. Chem. 249*, 257.
Deuel, T. F., and Stadtman, E. R. (1970), *J. Biol. Chem. 245*, 5206.
Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N. Y., Academic Press, pp 50–63.
Eisenberg, D., and Kauzmann, W. (1969), The Structure and Properties of Water, Fair Lawn, N. J., Oxford U. Press.
Feder, J., Garrett, L. R., and Wildi, B. S. (1971), *Biochemistry 10*, 4552.
Goldsack, D. E. (1970), *Biopolymers 9*, 247.
Howell, N., Akagi, J. M., and Himes, R. H. (1969), *Can. J. Microbiol. 15*, 461.
Hubbard, J. S., and Stadtman, E. R. (1967), *J. Microbiol. 93*, 1045.
Hunt, J. B., and Ginsburg, A. (1972), *Biochemistry 11*, 3723.
Kapoor, M., and Bray, D. (1968), *Biochemistry 7*, 3583.
Parfait, R. (1973), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 29*, 323.
Ravel, J. M., Humphreys, J. S., and Shive, W. (1965), *Arch. Biochem. Biophys. 111*, 720.
Rossmann, M. G., Adams, M. J., Buehner, M., Ford, G. C.,

Hackert, M. L., Liljas, A., Rao, S. T., Banaszak, L. J., Hill, E., Tsernoglou, D., and Webb, L. (1973), *J. Mol. Biol. 76,* 533.
Shapiro, B. M., and Stadtman, E. R. (1970), *Annu. Rev. Microbiol. 24,* 501.
Singleton, R., Jr., and Amelunxen, R. E. (1973), *Bacteriol. Rev. 37,* 320.
Stellwagen, E., Cronlund, M. M., and Barnes, L. D. (1973), *Biochemistry 12,* 1552.
Tanford, C. (1962), *J. Amer. Chem. Soc. 84,* 4240.
Valentine, R. C., Shapiro, B. M., and Stadtman, E. R. (1968), *Biochemistry 7,* 2143.
Wedler, F. C., and Boyer, P. D. (1972), *J. Biol. Chem. 247,* 993.
Wedler, F. C., and Hoffmann, F. M. (1973), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 32,* Abstr. 1408.
Wedler, F. C., and Hoffmann, F. M. (1974), *Biochemistry 13,* 3207.
Woolfolk, C. A., and Stadtman, E. R. (1967), *Arch. Biochem. Biophys. 118,* 736.

A Human Liver Aminopeptidase. The Amino Acid and Carbohydrate Content, and Some Physical Properties of a Sialic Acid Containing Glycoprotein†

Willis L. Starnes and Francis J. Behal*

ABSTRACT: An aminopeptidase has been purified to homogeneity from human liver. The amino acid analyses indicate that this enzyme is quite high in tryptophan content and that cysteine and cystine content are below the level of detection. Carbohydrate analyses show that the peptide portion of the enzyme is conjugated with sialic acid, neutral hexoses, and glucosamine, and that these residues make up about 17.5% of the dry weight of the purified enzyme. The sialic acid content, 4.14%, is unusually high for a carbohydrate-containing enzyme. In denaturing solvents, the purified enzyme exhibits a molecular weight near 118,000 (polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as well as in the presence of sodium dodecyl sulfate combined with urea, and sedimentation equilibrium in the presence of concentrated guanidinium chloride), and appears to behave ideally. In dilute salt solution, an equilibrium exists apparently between this species and a second species with a molecular weight near 235,000, presumably a dimer of the first species.

The aminopeptidases and particularly those enzymes of this group that catalyze the rapid hydrolysis of aminoacyl-β-naphthylamides have been the subject of continuing intensive study in our laboratory. Several enzymes belonging to this group of aminopeptidases have been identified in human tissues, and at least five distinct species separable by ion exchange chromatography are known to appear in blood (Behal *et al.*, 1964, 1965; Smith and Rutenburg, 1966; Panveliwalla and Moss, 1966). These five activities originate in specific organs, *i.e.*, one in the kidney, one in the duodenum, one in the liver, and two in the pancreas (Behal *et al.*, 1965).

This particular group of aminopeptidases is most active when Co^{2+} is present in the assay mixture (Thompson and Schwartz, 1959; Behal *et al.*, 1965, 1966, 1968; Behal and Little, 1968; and Behal and Story, 1969), although Co^{2+} is not the ion bound to the enzyme isolated from human liver (Garner and Behal, 1974). A broad range of substrate specificity is characteristic of this group of aminopeptidases, but aminoacyl-β-naphthylamides with nonpolar aminoacyl residues are preferred. L-Alanyl-β-naphthylamide is the most rapidly hydrolyzed substrate, and the enzyme has very low activity with acidic (*e.g.*, L-aspartyl-) and β-branched (*e.g.*, L-valyl-) β-naphthylamides. In addition, D-aminoacyl-β-naphthylamides are not hydrolyzed, a free α-amino group is required for enzymatic activity, and peptides are sequentially hydrolyzed from the N-terminus (Behal *et al.*, 1968). These features provide a unique opportunity to study a variety of enzyme-effector interactions.

While considerable information has been obtained about the use of human aminopeptidases in diagnosis of disease, little study has been directed toward defining the chemical and physical properties of these aminopeptidases.

This report describes some of the physical properties and the amino acid and carbohydrate content of an aminopeptidase isolated from human liver. A companion paper (Garner and Behal, 1974) describes the metal ion composition and role of metal ions in the activity of the enzyme.

Experimental Section

Materials

Except as otherwise indicated, all inorganic chemicals used were either Fisher, Baker and Adamson, J. T. Baker, or Mallinkrodt reagent grades. No differences resulted from the substitution of one brand for another. Fisher Chemical Co. was the source of β-naphthylamine hydrochloride, tris(hydroxymethyl)aminomethane (THAM, Tris), and *N*-(1-naphthyl)ethylenediamine dihydrochloride. Protein standards for gel electrophoresis and Sephadex G-200 chromatography were obtained from Worthington, Sigma Chemical Company, P-L Biochemicals, Inc., and Schwarz/Mann. Guanidinium chloride, *N,N,N′,N′*-tetramethylethylenediamine, Amido Schwarz, Coomassie Brilliant Blue, thioglycolic acid, dithiothreitol, neuraminidase, 2-mercaptoethanol, and sodium dodecyl sulfate

† From the Department of Biochemistry, Texas Tech University School of Medicine, Lubbock, Texas 79409. *Received February 11, 1974.* Supported in part by Research Grant D-529 from the Robert A. Welch Foundation, Houston, Texas 77002.