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PURIFICATION AND PROPERTIES OF GLUTAMINE SYNTHETASE FROM *BACILLUS STEAROTHERMOPHILUS*

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SUMMARY

Glutamine synthetase (EC 6.3.1.2) has been purified from *Bacillus stearothermophilus*. The molecular weight of the enzyme was found to be 630 000, and that of a component obtained on treating the enzyme with 4 M urea or 1% sodium dodecylsulfate plus 10 mM 2-mercaptoethanol 540 000 or 500 000, respectively, suggesting that the enzyme consists of 12 subunits. The enzyme requires divalent cations for activity, Mg^{2+} being the most effective activator. The pH and temperature optima in the presence of Mg^{2+} or Mn^{2+} are 7.3 or 6.5 and 70 or 75 °C, respectively. The enzyme is inactivated on exposure to 70 °C, but the inactivation is partially protected by Mg^{2+} (Mn^{2+} or glutamate) and completely by Mg^{2+} (Mn^{2+}), NH_4Cl and glutamate. Thermodynamic quantities for the enzyme reaction show a conformational transition at 58 °C. Glycine, alanine, serine, tryptophan, histidine, AMP and CTP inhibit the enzyme in the presence of Mg^{2+} or Mn^{2+}. The inhibition of the Mg^{2+}- or Mn^{2+}-activated enzyme by these compounds seems to be cumulative, except for the combined effects of amino acids on the Mn^{2+}-activated enzyme. Circular dichroism analyses of the enzyme show an α-helix, β-structure and unfolded conformation. Addition of Mg^{2+} or Mn^{2+} results in an increase of the α-helix content accompanied by a decrease of the unfolded conformation content.

INTRODUCTION

Brock [1] suggested that enzymes from thermophilic microorganisms possess a rigid protein conformation in order to gain thermostable properties by sacrificing the flexibility in enzyme conformation. Recent studies [2–4], however, have shown that thermophilic bacteria can produce regulatory enzymes which are both catalytically active and sensitive to allosteric regulation at high temperatures. The results indicate that thermostable regulatory enzymes retain flexibility in protein conformation at high temperatures. Investigations of the physicochemical, and especially thermal, properties of thermophile regulatory enzymes may afford information concerning the conforma-

tional requirement for maintaining allosteric interactions at high temperatures, as well as information concerning the thermophilicity or thermostability of thermophile enzymes. The synthesis of glutamine from glutamate and ammonia by glutamine synthetase (L-glutamate:ammonia ligase (ADP-forming), EC 6.3.1.2) represents a complex metabolic branch-point and imposes special requirements for the effective means of control [5]. In studies on enzymes from various mesophilic microorganisms [6, 7], evidence was presented for most organisms for a unique type of end-product control: cumulative feedback inhibition. As part of an investigation of thermophile allosteric enzymes, glutamine synthetase was purified from *Bacillus stearothermophilus*. Some of the catalytic and regulatory properties and the protein conformation of the enzyme are reported in the present study.

MATERIALS AND METHODS

Microorganism and culture

B. stearothermophilus NCA 2184 was aerobically cultured at 65 °C in the following medium: 4 g glucose, 4 g sodium L-glutamate, 0.1 g methionine, 5 g NaCl, 2 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 3 mg $MnCl_2 \cdot 4H_2O$, 10 μg biotin and 1 l of tap water; the pH was adjusted to 7.0 with 1 M NaOH. The cells cultured for 3.0–3.5 h (in a late logarithmic phase) were harvested. The cells at this stage of growth exhibited a maximum glutamine synthetase activity.

Assay of glutamine synthetase

During the purification of the enzyme, the activity was measured by the formation of γ-glutamylhydroxamate in the γ-glutamyl transfer reaction [8] at 65 °C. For the assay of the purified enzyme, the production of inorganic phosphate (P_i) in the biosynthetic reaction was measured. The biosynthetic reaction mixture (total volume, 0.5 ml) contained 7.5 mM ATP, 60 mM sodium glutamate, 20 mM NH_4Cl, 50 mM $MgCl_2$ or 7.5 mM $MnCl_2$ and 0.05–0.1 μg enzyme in 0.1 M imidazole–HCl buffer (pH 7.0). The reaction was initiated by the addition of enzyme at 65 °C and terminated by the addition of 0.5 ml of 3 % $HClO_4$. The liberated P_i was determined by the method of Peel and Loghman [9]. The activity was expressed as μmoles P_i formed per min per mg protein, except where indicated. The P_i formation under the experimental conditions used in the present study was linear with time for at least 14 min.

Protein determination

The protein concentration was determined by the method of Lowry et al. [10], using bovine serum albumin as a standard.

Ultracentrifugal analysis

Sedimentation velocity experiments were performed at 20 °C in a Hitachi ultracentrifuge, UCA-1, with phase-plate schlieren optics. The molecular weight of the native enzyme or the subunit obtained on treating the enzyme with 4 M urea at 4 °C for 10 h was estimated by the Archibald method [11] or by the meniscus depletion method as developed by Yphantis [12], respectively. The partial specific volume for the native enzyme or the subunit was calculated to be 0.735 from the amino acid composition (Table II), according to the method of Schachman [13].

Electrophoretic analysis

Disc-gel electrophoresis of the enzyme was carried out according to the procedures of Ornstein [14] and Davis [15] at 5% gel concentration. The polyacrylamide-gel electrophoresis of the enzyme in the presence of 0.1% sodium dodecylsulfate and 10 mM 2-mercaptoethanol was carried out according to the method of Weber and Osborn [16] at 7.5% gel concentration.

Amino acid analysis

The hydrolysis of the protein was carried out at 110 °C with 6 M HCl, in a sealed tube, under N_2. Analysis was carried out in a Hitachi liquid chromatogram, 334-0004.

Circular dichroism (CD)

The CD measurements were performed at room temperature in a Jasco automatic recording spectrophotometer, model J-20, with a CD attachment. The mean residue weight, M_0, of the enzyme was assumed to be 110, as estimated by the amino acid composition.

Infrared absorption spectra

Infrared absorption spectra of the enzyme in ^2H_2O were measured in a Jasco model IR-G infrared spectrometer.

Chemicals

ATP and AMP were purchased from Kyowa Hakko Co., and other nucleotides from Boehringer Mannheim Co. Bovine serum albumin, egg albumin, asparaginase (EC 3.5.1.1), trypsin (EC 3.4.4.4) and ribonuclease (EC 2.7.7.11) were obtained from Sigma Chemical Co.

RESULTS

Purification of the enzyme

Step I: Preparation of crude extracts. The cells (100 g, wet wt) suspended in 100 ml of 50 mM Tris–HCl buffer (pH 7.2) containing 10 mM $MgCl_2$ (Tris buffer) were disrupted in a French pressure cell and centrifuged at 15 000 × *g* for 20 min. The supernatant was further centrifuged at 100 000 × *g* for 40 min, and the resulting supernatant was diluted with Tris buffer to a protein concentration of 30 mg/ml.

Step II: Acid $(NH_4)_2SO_4$ fractionation. The pH of the crude extract was adjusted to 5.2 with 1 M acetic acid, and the precipitates formed after 15 min of standing of the mixture were removed by centrifugation. The supernatant was brought to 30% saturation with respect to $(NH_4)_2SO_4$, and then the pH was adjusted to 4.4 with 1 M acetic acid. The precipitates were collected by centrifugation, resuspended in Tris buffer containing 50 mM NaCl, and dialyzed against the same buffer. The procedure was repeated twice.

Step III: DEAE-Sephadex A-50 column chromatography. The dialyzed protein sample (2.1 g protein) was passed through a column of DEAE-Sephadex A-50 (3.6

cm × 18 cm) previously equilibrated with Tris buffer containing 50 mM NaCl. The column was washed with twice the column volume of Tris buffer containing 0.3 M NaCl. The enzyme was eluted by a linear gradient system of 0.3–0.5 M NaCl in Tris buffer, and fractions of 15 ml were collected. The fractions 18–25 were combined, concentrated through a collodion bag and dialyzed against Tris buffer containing 0.1 M NaCl.

Step IV: *Rechromatography on DEAE-Sephadex A-50.* The sample solution thus obtained (170 mg protein) was applied to a column of DEAE-Sephadex A-50 (2.1 cm × 30 cm) previously equilibrated with Tris buffer containing 0.1 M NaCl. The enzyme was eluted by a linear gradient system of 0.25–0.45 M NaCl in Tris buffer. Fractions of 15 ml were collected. The fractions 26–32 were collected and concentrated.

Step V: *Gel filtration on Bio-gel A-1.5 m.* The sample thus obtained (135 mg protein) was subjected to gel filtration on a column of Bio-gel A-1.5 m (2.0 cm × 90 cm). The buffer used for equilibration and development of the column was 50 mM Tris–HCl buffer (pH 7.2). Fractions of 10 ml were collected. The fractions 13–16 were combined, concentrated to a protein concentration of 10 mg/ml, and stored at −20 °C.

All the procedures were carried out at 4 °C.

The enzyme thus purified was homogeneous, ultracentrifugally and electrophoretically (Fig. 1). The purification scheme is presented in Table I.

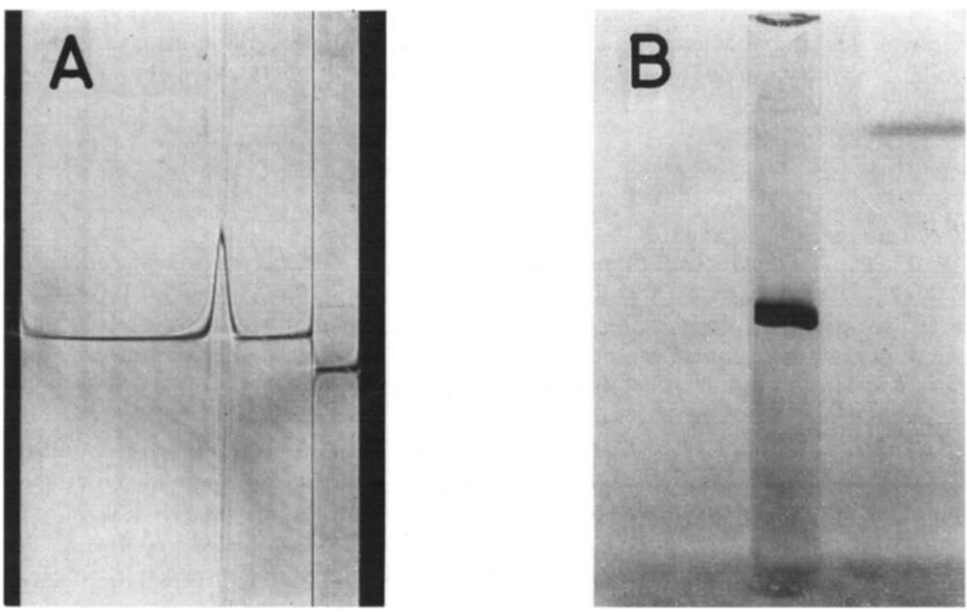

Fig. 1. Homogeneity of thermophile glutamine synthetase. A. Sedimentation pattern of the enzyme. The protein concentration was 0.4% in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl. The photograph was taken 20 min after reaching full sp ed, 51 200 rev./min. B. Disc electrophoretic pattern of the enzyme. The sample contained 50 μg protein in 0.5 M sucrose. The electrophoresis was carried out at 4 °C for 45 min at 5 mA/tube.

TABLE I

PURIFICATION SCHEME FOR GLUTAMINE SYNTHETASE FROM *B. STEAROTHERMOPHILUS*

Total activity was expressed in μmoles of γ-glutamylhydroxamate formed/min, and specific activity in μmoles of the compound formed per min per mg protein.

Stage	Protein (mg)	Total activity	Specific activity	Yield (%)
Crude extract	12 300	45 000	3.5	100
1st acid and $(NH_4)_2SO_4$ fractionation	4 400	39 000	8.8	87
2nd acid and $(NH_4)_2SO_4$ fractionation	2 100	34 000	16	76
1st DEAE-Sephadex chromatography	170	20 000	120	44
2nd DEAE-Sephadex chromatography	135	18 000	135	40
Bio-gel A-1.5 m gel filtration	80	13 000	160	29

Some hydrodynamic properties

The enzyme was found not to contain any metal ions such as Mg^{2+}, Mn^{2+} and Co^{2+}, as revealed by the atomic absorption method. The molecular weight of the enzyme was estimated to be 630 000, which was almost similar to those of the enzymes from *Escherichia coli* (mol. wt = 592 000) [17] and *Bacillus subtilis* (600 000) [18]. The sedimentation constant ($s^0_{20,w}$) was 18.5 S.

The enzyme, when treated with 4 M urea, was split into one component of molecular weight of 54 000. The enzyme ,when treated with 1% sodium dodecylsulfate and 10 mM 2-mercaptoethanol, was split into one component of molecular weight of 50 000 (Fig. 2). As in the case of the *E. coli* [19] and *B. subtilis* [17] enzymes, the thermophile enzyme may be considered to be composed of twelve subunits of equal molecular size.

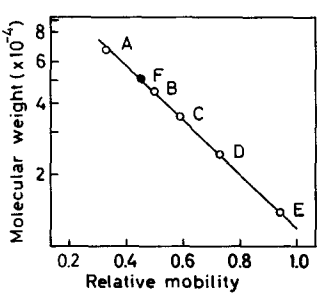

Fig. 2. Determination of the molecular weight of the subunit of thermophile glutamine synthetase in 0.1% sodium dodecylsulfate and 10 mM 2-mercaptoethanol polyacrylamide gels. A, bovine serum albumin; B, egg albumin; C, asparaginase; D, trypsin; E, ribonuclease; and F, thermophile glutamine synthetase. Each 50 μg of standard protein or of the enzyme was incubated at 37 °C overnight in the presence of 1% sodium dodecylsulfate and 10 mM 2-mercaptoethanol before the electrophoresis.

Ultraviolet absorption spectrum

The ultraviolet absorption spectrum of the enzyme exhibited a peak at 280 nm and a small shoulder around 290 nm. The 290-nm shoulder has not been reported for the *E. coli* [8] and *B. subtilis* [18] enzymes.

The ratio of $A_{289\ nm}/A_{260\ nm}$ for the thermophile enzyme was 1.9. The ratio reported for the non-adenylated glutamine synthetase from *E. coli* was 1.9, while the ratio for the adenylated enzyme was 1.4 (ref. 20). The thermophile enzyme may be considered to be not adenylated.

Amino acid composition

Any remarkable difference in amino acid composition was not observed between the thermophile and mesophile enzymes [8, 9] (Table II). The similarity of the amino acid composition of thermophile enzyme to that of the corresponding mesophile enzyme has been shown with other enzymes from the same thermophilic bacterium [21–23].

TABLE II

AMINO ACID COMPOSITION OF THE THERMOPHILE GLUTAMINE SYNTHETASE

Amino acid residues	Percentage by weight of residues*	Residues/minimum mol. wt of 50 000	
		Calculated	Nearest integer
Aspartic acid	10.5	46.1	46
Glutamic acid	12.9	50.2	50
Serine	3.4	20.0	20
Glycine	4.4	39.1	39
Alanine	6.7	47.4	47
Valine	5.6	28.4	28
Leucine	9.4	41.8	42
Isoleucine	5.7	25.1	25
Cysteine (1/2)**	0.9	4.7	5
Methionine	1.3	5.1	5
Proline	3.6	18.6	19
Phenylalanine	7.0	24.2	24
Tyrosine	5.5	16.7	17
Histidine	3.4	12.6	13
Lysine	6.8	26.6	27
Arginine	5.9	19.1	19
Tryptophan***	1.8	5.1	5
Threonine	4.7	23.2	23

* The values, except for cysteine and tryptophan, were those calibrated from the values obtained after 24, 48 and 72 h of hydrolysis, according to the method of Smith and Stockel [24].

** Cysteine was determined as cysteic acid after performic acid oxidation [25].

*** Tryptophan was determined photometrically according to the method of Goodwin and Morton [26].

Requirement of divalent cations

As in the cases of mesophile enzymes [6, 7, 27, 28], the thermophile enzyme required divalent cations for the activity, and among the cations tested (Mg^{2+}, Mn^{2+},

Co^{2+} and Ca^{2+}), the enzyme was most activated by Mg^{2+} and, to a lesser extent, by Mn^{2+}. The Mg^{2+}- and Mn^{2+}-activated enzymes exhibited the activities of 280 and 30 μmoles P_i formed per min per mg protein, respectively. When Mg^{2+} was added at the concentrations below 5 mM, no activation of the enzyme was observed. The activity increased with increasing Mg^{2+} concentrations above 5 mM and reached a constant level at 40–50 mM Mg^{2+}. Optimal Mn^{2+} concentration, on the other hand, was dependent on the ATP concentration as in the case of the *B. subtilis* enzyme [28], and at each concentration of ATP examined, maximum activity was observed when the concentration ratio of Mn^{2+} to ATP was about one. Inability to activate the enzyme at low concentrations as observed with Mg^{2+} was not demonstrated with Mn^{2+}.

Substrate specificity

The relative activities of the enzyme to various analogues of L-glutamate, ammonia and ATP in the presence of Mg^{2+} were essentially similar to the corresponding activities reported for the enzymes from *E. coli* [8] and *B. subtilis* [27] (Table III).

TABLE III

SUBSTRATE SPECIFICITY OF THE THERMOPHILE GLUTAMINE SYNTHETASE

Substrate*	Activity**	
	Mg^{2+}	Mn^{2+}
NH_4Cl	100 (100)	100 (100)
Methylamine	6 (—)	77 (—)
Hydroxylamine	30 (30)	74 (30)
ATP	100 (100)	100 (100)
GTP	30 (0)	58 (29)
CTP	0 (0)	0 (5)
UTP	0 (0)	81 (18)
ITP	9 (—)	63 (—)
L-Glutamate	100 (100)	100 (100)
D-Glutamate	11 (0)	86 (23)
DL-Glutamate	75 (—)	96 (—)
DL-α-Methylglutamate	10 (—)	91 (—)

* NH_4Cl, methylamine and hydroxylamine were used at 20 mM, L-glutamate and its analogues at 60 mM, and nucleotides at 7.5 mM, respectively, in final concentrations.

** The activity was expressed as a percentage versus that measured with NH_4Cl, ATP and L-glutamate as substrates, both in the presence of $MgCl_2$ and $MnCl_2$. The values in parentheses are those reported for the *B. subtilis* enzyme [27].

GTP, ITP and D-glutamate, which were not hydrolyzed by the *B. subtilis* enzyme at all, however, were slightly hydrolyzed by the thermophile enzyme. The thermophile enzyme in the Mn^{2+} system was able to utilize all the compounds tested, except CTP, and the compounds were much better substrates for the thermophile enzyme than for the *B. subtilis* enzyme [27].

Effect of pH

The pH optimum for the thermophile enzyme was 7.8 with 10 mM Mg^{2+} and 7.3 with 50 mM Mg^{2+}. On the other hand, the pH optimum with 4–10 mM Mn^{2+} was

constant at pH 6.5. The feature that the Mn^{2+}-activated enzyme has a more acidic pH optimum than the Mg^{2+}-activated enzyme has also been observed with mesophile enzymes [8, 27].

Some kinetic properties

The values of the Michaelis constant (K_m) for glutamate, NH_4Cl and ATP at 65 °C were 8.3, 0.67 and 3.3 mM with 50 mM Mg^{2+} and 2.0, 0.15 and 3.5 mM with 10 mM Mn^{2+}, respectively. The values of maximum velocity (V) for these substrates at 65 °C were 0.10, 0.11 and 0.14 μmole P_i per min per mg protein with 50 mM Mg^{2+} and 0.0051, 0.011 and 0.011 with 10 mM Mn^{2+}, respectively. As shown in Fig. 3, either in the Mg^{2+} and Mn^{2+} system, the curve representing the relationship between the logarithm of K_m and the reciprocal of temperature ($1/T$) for each substrate was a straight line in the temperature range from 40 to 70 °C. On the other hand, the curves representing the relationships between $\log(1/V)$ and $1/T$ for the three substrates, both in the Mg^{2+} and Mn^{2+} systems, were all composed of two straight lines intersecting at 58 °C. The results may indicate that the activation energy for the enzyme reaction with reference to each of the substrates was different below and above 58 °C, either in the Mg^{2+} or the Mn^{2+} system. This may indicate a thermal transition in the conformation of the enzyme at 58 °C, as suggested for other enzymes from the same bacterium [21–23, 29].

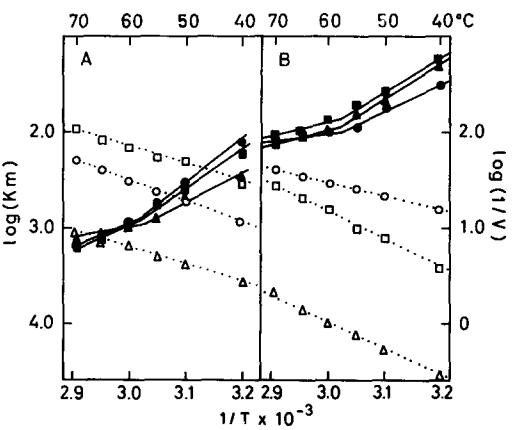

Fig. 3. Effects of temperature on K_m and V in the Mg^{2+} and Mn^{2+} systems. The reaction mixture contained 50 mM $MgCl_2$ (A) or 7.5 mM $MnCl_2$ (B). ·····, log (K_m); ———, log ($1/V$). ○···○ and ●—●, ATP; □···□ and ■—■, L-glutamate; and △···△ and ▲—▲, NH_4Cl. K_m was expressed in M.

Effect of temperature on the activity

The optimum temperature for the Mg^{2+}-activated enzyme was 70 °C and that for the Mn^{2+}-activated enzyme, 75 °C. Both the enzymes completely lost their activities above 80 °C.

The thermophile enzyme was stable at −20 °C for at least 8 months, for one week at 0–4 °C and for at least 5 h at 4–50 °C. When the enzyme (10 μg) was incubated in 20 mM Tris–HCl buffer (pH 8.0) at 70 °C, however, the activity decreased

rapidly; 90% loss after 3 h and 100% loss after 5 h. On exposure to ATP (7.5 mM) or NH_4Cl (20 mM) to 70 °C, the enzyme became more heat labile; 100% loss after 3 h. The heat inactivation of the enzyme was partially protected by Mg^{2+} (50 mM) or glutamate (60 mM); 40% loss after 5 h. The enzyme was more strongly protected by the combination of Mg^{2+} and glutamate (or NH_4Cl); only 5% loss after 5 h. The inactivation was completely protected by the combination of Mg^{2+}, glutamate and NH_4Cl. The effects of Mg^{2+} on the thermostability of the enzyme were completely replaced by 7.5 mM Mn^{2+}.

Effects of feedback inhibitors on the activity

Maximum inhibition by each of the inhibitors listed in Table IV on mesophile enzymes [7, 27, 30] was generally obtained at 1–5 mM. The concentrations of the amino acids exhibiting maximum inhibitory effects on the thermophile enzymes, on

TABLE IV

EFFECTS OF INHIBITORS ON THE THERMOPHILE GLUTAMINE SYNTHETASE

The following inhibitor concentrations were used: 20 mM amino acids and 10 mM nucleotides. The activity was expressed as a percentage against the activity without inhibitor.

Inhibitor	Activity (%)			
	Mg^{2+}		Mn^{2+}	
	Observed	Calculated for cumulative inhibition*	Observed	Calculated for cumulative inhibition*
None	100		100	
Alanine	50		30	
Glycine	70		30	
Serine	60		30	
Tryptophan	85		80	
Histidine	95		90	
Glutamine	85		65	
Glycine + serine	43	42	—	—
Alanine + glycine	—	—	32	9
Alanine + glycine + serine	22	18	22	3
Alanine + glycine + serine + tryptophan	26	25	18	4
AMP	50		60	
CTP	50		35	
AMP + CTP	28	25	25	21

* Calculated according to the method of Stadtman [31].

the other hand, were 15–20 mM. The inhibitory effects of the nucleotides on the thermophile enzyme increased with increasing their concentrations, and no saturation of the inhibition degree was observed at least up to 15 mM. In the present study the amino acids and nucleotides were used at 20 and 10 mM, respectively, although the concentrations appeared to exceed the physiological level.

As in the cases of mesophile enzymes [7, 27, 30], the Mg^{2+}-activated enzyme appeared to be inhibited cumulatively by the amino acids and nucleotides (Table IV).

The Mn^{2+}-activated enzyme also seemed to be cumulatively inhibited by the nucleotides. The combined effect of the amino acids on the Mn^{2+}-activated enzyme, however, may not be considered to be cumulative. The results may suggest that the two enzymes may have different regulatory properties with reference to the amino acids.

Circular dichroism

The CD spectrum of the enzyme exhibited two bands at 210 and 216 nm in the ultraviolet region (Fig. 4). On the addition of 10 mM Mg^{2+}, no change in the CD spectrum was observed. The spectrum, however, changed gradually with increasing Mg^{2+} concentrations above 10 mM and the change stopped at 50 mM Mg^{2+} or above; the 210- and 216-nm bands shifted to 208 and 220 nm, respectively. The results indi-

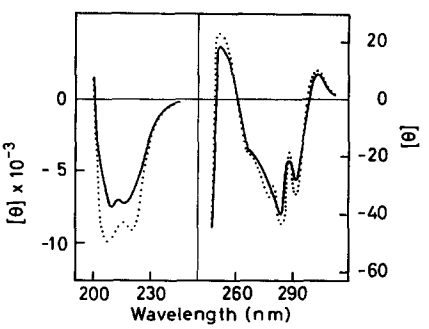

Fig. 4. CD spectra of thermophile glutamine synthetase in the near ultraviolet and ultraviolet regions in the absence and presence of 50 mM $MgCl_2$. The solid curves were for the enzyme without Mg^{2+} and the dashed lines for that with Mg^{2+}. The absorbance of the sample at each wavelength was always kept below 2. The solvent used was 20 mM Tris–HCl buffer (pH 7.2).

cate that the folded conformation (α-helix and β-structure) may be present in the enzyme molecule and the fraction of the folded conformation increased on the addition of increasing concentrations of Mg^{2+}. The presence of the α-helix and β-structure in the enzyme molecule was confirmed by the infrared absorption spectrum, since the spectrum exhibited the bands of an amide I band at 1650 cm^{-1} (α-helix) and 1690 and 1630 cm^{-1} (β-structure), besides the bands at 1655 and 1640 cm^{-1} (unfolded conformation) [32, 33].

The fractions of α-helix, β-structure and unfolded conformation of the enzyme, as estimated from the CD data according to the method of Chen et al. [34] were 7, 34 and 59 % and those of the enzyme with 50 mM Mg^{2+} were 20, 32 and 48 %, respectively. On the addition of Mn^{2+}, the CD spectrum of the enzyme changed similarly, although the change was proportional to the Mn^{2+} concentrations and stopped at 10 mM. The fractions of α-helix, β-structure and unfolded conformation of the enzyme with 10 mM Mn^{2+} were 14, 37 and 49 % ,respectively.

The enzyme exhibited an optical activity in the near ultraviolet region, and on addition of 50 mM Mg^{2+} the CD spectrum in this region changed slightly as shown in Fig. 4. When considered the band positions (270, 284 and 294 nm), the results suggest that the states of the aromatic amino acid residues in the enzyme molecule may be slightly changed by Mg^{2+}.

DISCUSSION

The protein structure of the glutamine synthetase purified from *B. stearothermophilus* seemed to be similar to those of the enzymes from mesophilic bacteria, *E. coli* [8, 17, 19, 30] and *B. subtilis* [18], in molecular size, subunit structure and amino acid composition. The thermophile enzyme, however, was found to be different from the mesophile enzymes in the effects of divalent cations on protein structure. The *E. coli* enzyme was shown to contain Mn^{2+} (ref. 17). The enzymes from some mesophilic bacteria such as *Bacillus cereus* and *Bacillus licheniformis* were reported to be unstable in the absence of Mn^{2+} (ref. 7). The thermophile enzyme, on the other hand, contained no metal ion and was stable at the temperatures below 50 °C even in the absence of Mn^{2+} or Mg^{2+}. The *E. coli* enzyme exhibited no optical profile in the near ultraviolet region [35] whereas the thermophile enzyme exhibited optical activity probably attributed to aromatic amino acid residues in that region. In addition, the states of aromatic amino acid residues may be considered to be changed slightly on addition of Mg^{2+}, as suggested from the CD spectra of the enzyme with and without Mg^{2+}. Clearly demonstrated by the CD analyses of the enzyme was a change in the secondary structure of the enzyme on the addition of Mg^{2+} or Mn^{2+}: the α-helical content of the enzyme increased and the content of unfolded conformation decreased.

The thermophile enzyme was stable at 50 °C for 5 h, but was completely inactivated at 70 °C after 3 h. The inactivation at 70 °C of the enzyme, however, was partially protected by Mg^{2+} or Mn^{2+}. Some conformational change accompanied by such a change in the secondary structure of the enzyme may be related to the thermostability of the enzyme. The Mg^{2+} of the concentrations below 5 mM was unable to activate the thermophile enzyme, but the activity increased with an increase in the Mg^{2+} concentration above 5 mM and reached a constant level at 40–50 mM Mg^{2+}. No change in the secondary structure of the enzyme was either observed with up to 10 mM Mg^{2+}, and above 10 mM the structural change proceeded with an increase in the Mg^{2+} concentration and stopped at 50 mM Mg^{2+}. In the Mn^{2+} system, the enzyme activity was proportional to the Mn^{2+} concentration and the structural change was observed even at low concentrations of Mn^{2+}. The activation of the thermophile enzyme may also be attributable to some change in the enzyme conformation accompanied by such a secondary structural change.

The effects of Mn^{2+} on the thermostability and secondary structure of the thermophile enzyme were essentially similar to those of Mg^{2+}. The Mn^{2+}-activated enzyme, however, was different from the Mg^{2+}-activated enzyme in specific activity and pH and temperature dependencies. In addition, the Mg^{2+}-activated enzyme seemed to be inhibited cumulatively by glycine, alanine and serine, while the combined effect of the amino acids on the Mn^{2+}-activated enzyme was not considered to be cumulative. It is suggested, therefore, that the Mg^{2+}- and Mn^{2+}-activated enzymes may be different not only in the conformation around the active site but also in the structural requirement for maintaining the allosteric interaction of the enzyme.

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