Purification and Characterization of Glutamine Synthetase from *Clostridium pasteurianum*†

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ABSTRACT: Glutamine synthetase from *Clostridium pasteurianum* grown on molasses as the sole carbon source and ammonium chloride as the nitrogen source has been purified to homogeneity (45-fold) with 32% recovery. The procedure involves ammonium sulfate precipitation and chromatography on a combined Sepharose 4B/DEAE-Sephadex A-50 column. The purified enzyme being very unstable was stabilized by the addition of 25% (v/v) glycerol. The enzyme has an unusually high molecular weight of 1×10^6 and 20 subunits of M_r 50 000 each, as determined by gel filtration and sodium dodecyl sulfate gel electrophoresis, respectively. It has an absorption maximum at 280 nm and a fluorescence emission maximum at 380 nm when excited at 280 nm. Its substrate binding pattern as studied by fluorescence quenching studies is different from that of the *Escherichia coli* enzyme. Both the γ-glutamyltransferase and synthetase activities reside in the same protein as the ratio of the two activities at each step of purification remains constant and the enzyme exhibits optimal transferase and synthetase activities at the same pH (7.2) and temperature (50 °C). The thermal stabilities of both activities were also similar, and decay of both the activities at 50 °C ran parallel. The enzyme shows stabilization by substrates, as L-glutamate, Mg^{2+}, and ATP + Mg^{2+} protected both the synthetase and γ-glutamyltransferase activities against thermal inactivation. Storage in 25% (v/v) glycerol enhanced the thermal stability of glutamine synthetase. Metal ion requirement and substrate specificity of the enzyme have been examined. Maximum synthetase activity occurs when $[Mg^{2+}]$:[ATP] = 2. The K_m^{app} values are as follows (in parentheses): ATP (0.34 mM), NH_2OH (0.4 mM in the synthetase reaction and 4.1 mM in the transferase reaction), glutamine (14.7 mM), ADP (3.8×10^{-4} mM), arsenate (2.5 mM), and L-glutamate (3.4 mM, 22.2 mM). The enzyme exhibits negative cooperativity in the binding of glutamate. Amino acids such as L-serine, glycine, L-alanine, and L-aspartic acid inhibit the enzyme.

In biological systems, ammonia assimilation is through the enzymatic conversion of ammonium salts to essential nitrogenous compounds. The most commonly occurring pathway in organisms is the reductive amination of α-ketoglutarate by glutamate dehydrogenase:

$$\alpha\text{-ketoglutarate} + \text{NAD(P)H} + \text{NH}_3 \rightleftharpoons \text{glutamate} + \text{NAD(P)}^+$$

However, this enzyme is absent in the free-living nitrogen fixers, such as *Clostridium pasteurianum* (Kleiner, 1979), *Azotobacter vinelandii* (Kleiner, 1975), and several species of blue green algae (Hoare et al., 1967). Early isotopic studies with *C. pasteurianum* (Zelitch et al., 1951) showed that while about 50% of the ^{15}N fixed accumulated in the culture medium as ammonia, which had the highest label, the other compound, with the second highest label, was glutamine. Consistent with this observation, the glutamine synthetase (EC 6.3.1.2)/glutamate synthase (EC 1.4.1.14) pathway was detected in *C. pasteurianum* (Dainty & Peel, 1970; Dainty, 1972).

$$\text{L-glutamate} + \text{ATP} + \text{NH}_3 \xrightarrow[M^{2+}]{\text{glutamine synthetase}} \text{L-glutamine} + \text{ADP} + \text{P}_i$$

$$\text{L-glutamine} + \alpha\text{-ketoglutarate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{glutamate synthase}} 2\ \text{glutamate} + \text{NAD}^+$$

This pathway is appropriate for diazotrophs as they are by definition ammonia-limited, and glutamine synthetase, which has a low K_m for NH_3, enables the organism to assimilate ammonia at such low levels as those produced by nitrogenase reaction. So low is this K_m for ammonia that glutamine synthetase has been proposed to have an ammonia scavenging role in metabolism (Umbarger, 1969). In addition to this role, glutamine synthetase catalyzes the biosynthesis of glutamine, which is a compound of central importance in nitrogen metabolism (Stadtman, 1973). Glutamine synthetase, as the first enzyme in this pathway, has a key position and modulates the overall flow of NH_4^+ to organic nitrogen. Thus, glutamine synthetase is important as a target of cellular control and also from the standpoint of reaction mechanism, as it catalyzes a multisubstrate reaction. To understand its regulatory role and the mechanism of action of glutamine synthetase, the molecular properties of the enzyme should be determined.

In the case of nitrogen-fixing bacteria, glutamine synthetase has been purified to homogeneity from *A. vinelandii* (Kleinschmidt & Kleiner, 1978; Lepo et al., 1979; Siedel & Shelton, 1979), but except for preliminary studies (Kleiner, 1979; Hubbard & Stadtman, 1967), purification of the enzyme from *C. pasteurianum*, which is the most active anaerobic nitrogen fixer and hence an organism of choice for investigating nitrogen fixation, has not been reported. In this paper, we report the purification and properties of glutamine synthetase from *C. pasteurianum*.

Materials and Methods

Chemicals. α-Ketoglutarate, 2-mercaptoethanol, L-glut-

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amine, ADP,[1] ATP, bovine serum albumin, γ-glutamyl hydroxamate, Coomassie Brilliant Blue R-250, tris(hydroxymethyl)aminomethane, and biotin were obtained from Sigma Chemical Co. (St. Louis, MO); EDTA, magnesium chloride, manganese chloride, bromophenol blue, ammonium sulfate, and ammonium chloride were supplied by BDH (Poole, England); L-glutamic acid, bis[*N*,*N*′-methylenebis(acrylamide)], ammonium persulfate, ethylenediamine, glycerol, hydroxylamine hydrochloride, and acrylamide were purchased from E. Merck (Darmstadt, West Germany); *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was procured from Koch Light Labs (Colnbrook, England); sodium dodecyl sulfate (SDS) and the molecular weight markers lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and phosphorylase *b* were from Bio-Rad Laboratories (CA), while the molecular weight markers ferritin, catalase, aldolase, and bovine serum albumin were from Boehringer Mannheim (Mannheim, West Germany); thyroglobulin (bovine thyroid), DEAE-Sephadex A-50, and Sepharose 4B were acquired from Pharmacia Fine Chemicals (Uppsala, Sweden); disodium hydrogen arsenate was from Riedel-DeHaeu Ag Seelze (Hanover, West Germany). All other chemicals were of the highest purity available locally from other sources. All solutions were prepared in double-distilled deionized water.

Organism and Cultivation. *C. pasteurianum* (ATCC no. 6013) was obtained from American Type Culture Collection Center, Washington, DC. The soil spore stock was revived by heat shock and was grown on potato medium in 2% (w/v) sucrose solution under anaerobic conditions at 37 °C for 48–50 h. In the synthetic medium, sucrose was replaced by molasses as the sole carbon source and contained (in mg/L) molasses (sucrose content: 20000), $MgSO_4 \cdot 7H_2O$ (25), iron(III) citrate (9), $MnSO_4$ (0.2), $Na_2MoO_4 \cdot 2H_2O$ (0.15), $CaCl_2 \cdot 2H_2O$ (25), biotin (20), K_2HPO_4 (15600), KH_2PO_4 (1400), and NH_4Cl (212).

Molasses (50% sucrose) was diluted with an equal volume of distilled water, and the pH of its solution was adjusted to 4.0 with 2 N H_2SO_4. It was then heated to 80 °C in a water bath for 10 min. The precipitate formed was centrifuged off, and the supernatant, whose pH was adjusted to 7.0 with 2 N NaOH, was then used for cell growth. The rest of the bacterium growth procedure was same as that reported by Rosenblum and Wilson (1950).

The pH of the medium was maintained between 6.5 and 7.0 with 10 N KOH during cell growth, and the cells were harvested in the late log phase in a Sharples centrifuge, washed twice with cold 50 mM potassium phosphate buffer, pH 7.2, and stored frozen at −22 °C. The purity of the *C. pasteurianum* cells was checked occasionally by the method of Topley and Wilsons (1975).

Buffer A. Buffer A was 200 mM potassium phosphate, pH 7.2. Buffer B was buffer A containing α-ketoglutarate (10 mM), 2-mercaptoethanol (5 mM), and EDTA (1 mM).

Preparation of Cell-Free Extract. The cells (1 g wet weight/10 mL) were suspended in buffer B and disrupted for 2 min with an MSE ultrasonic oscillator (20 kHz) at 0–4 °C. Cell debris was removed by centrifuging at 20000*g* for 30 min at 4 °C.

Enzyme Assays. The enzyme was assayed by the following reactions.

(*a*) *Synthetase Reaction*:

$$\text{L-glutamate} + \text{ATP} + NH_2OH \xrightarrow{Mg^{2+}} \gamma\text{-glutamyl hydroxamate} + \text{ADP} + P_i$$

The assay solution contained the following reagent solutions (in mM) whose pH had been adjusted to 7.2: Tris-HCl buffer (20), L-glutamate (90), ATP (4), $MgCl_2 \cdot 6H_2O$ (12), and NH_2OH (60), in a total volume of 2.0 mL. The reaction was carried out at 50 °C, and it was initiated by the addition of enzyme preparation. The reaction was stopped after 10 min by the addition of 0.5 mL of a solution containing equal volumes of 24% trichloroacetic acid, 6 N HCl, and 10% $FeCl_3 \cdot 6H_2O$ in 0.02 N HCl. The absorbance of the γ-glutamyl hydroxamate formed in the reaction mixture was measured after 10 min at 510 nm against a control containing all reagents except L-glutamate. The amount of γ-glutamyl hydroxamate produced was determined from a reference curve prepared from pure γ-glutamyl hydroxamate.

(*b*) *γ-Glutamyltransferase Reaction*:

$$\text{L-glutamine} + NH_2OH \xrightarrow[AsO_4^{3-},\ \text{ADP}]{Mn^{2+}} \gamma\text{-glutamyl hydroxamate} + NH_3$$

The assay solution contained the following reagent solutions (in mM) whose pH had been adjusted to 7.2: Tris-HCl (20), L-glutamine (30), sodium ADP (0.4), sodium arsenate (20), $MnCl_2 \cdot 4H_2O$ (3), and NH_2OH (60), in a total volume of 2.0 mL. The reaction was carried out at 30 °C for 10 min, and it was initiated by the addition of enzyme preparation. γ-Glutamyl hydroxamate formed in the reaction mixture was determined as discussed above, against a control containing all reagents except L-glutamine.

Specific Activity. Specific activity of the enzyme is defined as the number of micromoles of γ-glutamyl hydroxamate formed in 10 min per milligram of protein.

Inorganic Phosphate Determination. The purified enzyme in buffer B was dialyzed exhaustively against 100 mM Tris-HCl, pH 7.2, and was checked for the inorganic phosphate content by the method of Boyer et al. (1959) for its complete removal.

Protein Determination. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard protein. Protein concentration in chromatographic procedures was followed spectrophotometrically by the method of Warburg and Christian (1941) on a Pye Unicam SP 500 spectrophotometer.

Purification of Glutamine Synthetase. All steps were carried out at 0–4 °C. Purification steps were monitored by determining the γ-glutamyltransferase activity.

Ammonium Sulfate Fractionation. The cell-free extract (protein: 6.4 mg/mL) obtained by ultrasonication was brought to 30% saturation by adding finely powdered ammonium sulfate, with constant stirring. The pH of the solution was maintained between 6.9 and 7.2 by the addition of 1 N KOH. The solution was occasionally stirred and allowed to stand for 40 min and then centrifuged at 20000*g* for 30 min. The pellet was discarded, and the supernatant was brought to 50% saturation with addition of solid ammonium sulfate. As before, after 40 min, the solution was centrifuged at 20000*g* for 30 min. The pellet obtained was dissolved in buffer B and is hereafter referred to as the 30–50% ammonium sulfate fraction.

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; buffer A, 200 mM potassium phosphate buffer, pH 7.2; buffer B, buffer A containing α-ketoglutarate (10 mM), 2-mercaptoethanol (5 mM), and EDTA (1 mM); K_m^{app}, apparent Michaelis–Menten constant; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

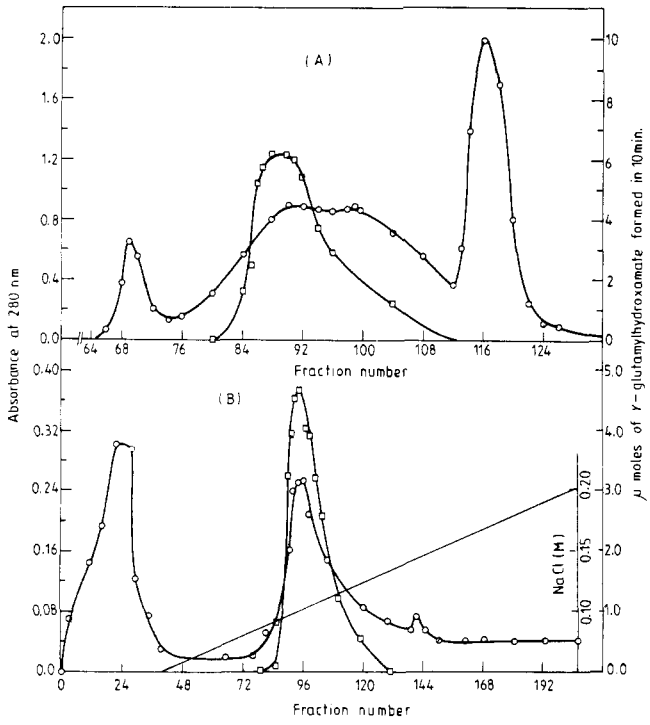

FIGURE 1: (A) Gel filtration of glutamine synthetase on Sepharose 4B. The ammonium sulfate precipitated protein (30–50% saturation) redissolved in buffer B was applied on to a Sepharose 4B column (2.2 × 90 cm) previously equilibrated with the same buffer. Fractions (3 mL) were collected at a flow rate of 15 mL/h, and their absorbance was measured at 280 nm (○). Aliquots (0.05 mL) were withdrawn from each fraction and assayed for γ-glutamyltransferase activity (□) as given in the text. (B) Ion-exchange chromatography of glutamine synthetase on DEAE-Sephadex A-50. When the glutamine synthetase activity appeared in the eluent (fraction 84 onward) of the Sepharose 4B column, the column was mounted on the preequilibrated DEAE-Sephadex A-50 column (2.2 × 35 cm) with a flow rate of 15 mL/h being maintained in both the columns. The sepharose 4B column was disconnected when 51 mL of the eluent from the DEAE-Sephadex A-50 column had been collected (that is, fractions 84–100 of the Sepharose 4B column). The DEAE-Sephadex A-50 column was then washed with buffer B until no protein was detected in the eluent. The enzyme was eluted with a linear gradient of 50–200 mM NaCl in 500 mL of buffer B, and the absorbance was measured at 280 nm (○). Aliquots (0.05 mL) were withdrawn from each fraction and assayed for γ-glutamyltransferase activity (□) as given in the text.

Combined Sepharose 4B/DEAE-Sephadex A-50 Chromatography. The 30–50% ammonium sulfate fraction (protein: 12.8 mg/mL) was directly loaded onto a Sepharose 4B column (2.2 × 90 cm) previously equilibrated with buffer B. When the glutamine synthetase activity started appearing in the eluent (from fraction 84 onward) (Figure 1A), the column was mounted on a DEAE-Sephadex A-50 column (2.2 × 35 cm) previously equilibrated with the same buffer, so that the eluent from the Sepharose 4B column was directly loaded on the ion-exchange column, without disturbing the top of the gel bed. A flow rate of 15 mL/h was maintained in both the columns. The Sepharose 4B column was disconnected as soon as 51 mL of the eluent from the DEAE-Sephadex A-50 column (17 fractions of 3 mL each) had been collected. The DEAE-Sephadex A-50 column was now connected to the buffer B reservoir and washed until no protein appeared in the eluent. The column was then eluted with a linear gradient of 50–200 mM NaCl in buffer B in a total volume of 500 mL. Glutamine synthetase activity eluted between fractions 85 and 120 (Figure 1B).

Electrophoretic Methods. Polyacrylamide disc gel electrophoresis was carried out according to the method of Davis (1964) using a Shandon SAE 2717 gel electrophoresis apparatus employing 7% and 5% gel in Tris–glycine buffer, pH 8.3. After completion of the run the gels were stained with Coomassie Brilliant Blue R-250 for 20 min and then destained with 7% acetic acid until the protein bands appeared.

Activity Staining. The gels obtained after electrophoresis as described above were immersed in γ-glutamyltransferase assay mixture containing reagents whose concentrations were twice those of the normal assay solution for 30 min at 30 °C. When the gels were removed, washed with distilled water, and transferred to the stop mixture, a brown band of γ-glutamyl hydroxamate appeared at a position corresponding to the band obtained with Coomassie Brilliant Blue R-250.

Ouchterlony Double-Immunodiffusion Test (Ouchterlony, 1949). Purified glutamine synthetase (0.8–1.0 mg) was mixed with an equal volume of Freund's incomplete adjuvant and was injected into three areas of the back of a rabbit. The process was repeated after 2 weeks. Further intradermal injections of the enzyme (protein: 0.8–1.0 mg in 200 mM potassium phosphate buffer, pH 7.4, containing 0.85% NaCl) were made at 2-week intervals, and blood was collected from an ear vein 7 days after each injection. Blood was allowed to clot, and the serum was collected by centrifugation at 10000*g* for 10 min and stored at 0 °C. Control serum was prepared from the blood of the same rabbit before immunization and stored at 0 °C. The Ouchterlony double-immunodiffusion test was done in a 2% agar plate containing phosphate-buffered saline. The antisera were placed in the central well, and antigen was placed in the peripheral well. Diffusion was allowed to occur for 24–48 h at 4 °C till the precipitin lines were clearly visible. Plates were washed with 0.85% NaCl for 24 h to remove nonprecipitated protein and were then photographed.

Determination of Molecular Weight. Molecular weight of the purified glutamine synthetase (protein: 2 mg) was determined by gel filtration on a Sepharose 4B column (1 × 112 cm) equilibrated with buffer B, at a flow rate of 30 mL/h. The void volume (V_0) of the column was determined with Blue Dextran. The column was calibrated with the marker proteins (protein: 2 mg/mL) thyroglobulin (M_r 669 000), ferritin (M_r 440 000), catalase (M_r 240 000), aldolase (M_r 158 000), and bovine serum albumin (M_r 68 000). Elution volume (V_e) for each protein was determined, and the ratio of elution volume to void volume (V_e/V_0) was plotted against molecular weight on a semilogarithmic scale. The molecular weight of glutamine synthetase (detected by assaying the enzyme activity) was estimated from the calibration graph.

The subunit molecular weight and also the homogeneity of the glutamine synthetase were determined by sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis in Tris buffer by the method of Weber and Osborn (1975). Marker proteins used were lysozyme (M_r 14 400), soybean trypsin inhibitor (M_r 21 500), carbonic anhydrase (M_r 31 000), ovalbumin (M_r 45 000), bovine serum albumin (M_r 66 200), and phosphorylase *b* (M_r 92 500). The markers were diluted (1:20) with buffer [10 mM Tris-HCl, pH 6.8, containing 0.1% SDS, 0.1% 2-mercaptoethanol, 10% (w/v) glycerol, and 0.001% bromophenol blue] and denatured by heating at 100 °C for 5 min before they were applied to the gel. The purified enzyme (protein: 0.4 mg/mL) was treated similarly. A current of 1 mA/tube was initially applied until the protein entered the gel, and then the current was raised to 3 mA/tube for 8 h. After the electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 for 12 h, then destained with 25% 2-propanol–10% acetic acid, restained with Coomassie

Table I: Purification of Glutamine Synthetase from *C. pasteurianum*

purification step	total volume (mL)	total act. (μmol of γ-glutamyl hydroxamate formed in 10 min)	total protein (mg)	sp act.	recovery (%)	purification (x-fold)
crude	100	9216	640	14.4	100	1
ammonium sulfate fractionation (30–50% saturation)	15	5472.1	190	28.8	59.3	2
combined Sepharose 4B/DEAE-Sephadex A-50 column chromatography	27	2989.2	4.6	650	32.4	45

Brilliant Blue R-250 for 20 min, and then destained with 7.5% acetic acid.

The molecular weights of the marker proteins were plotted against their relative mobilities (R_m) on a semilogarithmic scale, and the molecular weight of the subunits was determined from this plot.

Results and Discussion

Stability of Glutamine Synthetase in Crude Extracts under Different Storage Conditions. Glutamine synthetase in cell-free extracts was quite unstable; about 40% of the activity was lost within 24 h of storage. An attempt was made to stabilize the enzyme by addition of various stabilizing agents, such as EDTA, 2-mercaptoethanol, and α-ketoglutarate, individually and in different combinations. The results are shown in Figure 2. Though α-ketoglutarate and EDTA stabilized the enzyme individually, mercaptoethanol alone inactivated the enzyme. So they were tried in different combinations to see their cooperative effect on the stability. Though either EDTA or α-ketoglutarate alone stabilized the enzyme, yet a combination of mercaptoethanol plus EDTA and mercaptoethanol plus α-ketoglutarate hastened the inactivation. Peculiar as it may seem, mercaptoethanol (5 mM) in the presence of both α-ketoglutarate (10 mM) and EDTA (1 mM) improved the stability of the enzyme, and it retained 95% glutamine synthetase activity after 24 h. They were, therefore, routinely added to buffer A during purification.

Purification. The enzyme was purified 45-fold from the initial crude extract with a recovery of 32%. The results of the purification are summarized in Table I. The ratio of the γ-glutamyltransferase activity to synthetase activity was checked after each purification step and was found to be almost constant. These results suggested that the two enzyme activities are associated with the same protein.

An earlier procedure, which involved ammonium sulfate fractionation, overnight dialysis against buffer B, chromatography on Sepharose 4B followed by pooling and concentration of the fractions against sucrose, dialysis, and then loading on a DEAE-Sephadex A-50 column, resulted in only 11% recovery and 26-fold purification of glutamine synthetase (results not shown). The increased recovery (32%) achieved in the present procedure could be due to faster separation of proteolytic enzymes from the glutamine synthetase on Sepharose 4B when the ammonium sulfate fraction was directly applied to this column and dialysis of protein solution was avoided. This procedure also eliminates steps involving concentration and dialysis of the pooled Sepharose 4B fractions, which further reduces time for the action of proteolytic enzymes. So the present purification procedure reduces the time from 96 h to just 50 h and also improves recovery from 11% to 32%.

Tests for Homogeneity. The purified protein was subjected to various tests to verify the homogeneity of the preparation. Figure 3A shows the results of gel electrophoresis under nondenaturing conditions, which yielded a single band at pH 8.3 on 7% (photograph not shown) and 5% polyacrylamide gel.

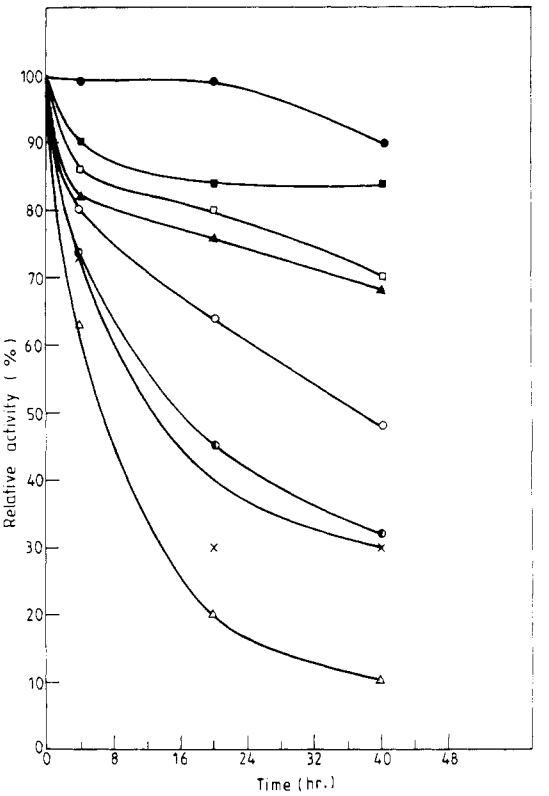

FIGURE 2: Stability of glutamine synthetase in cell-free extracts stored under different conditions. Cell-free extract (protein: 7 mg/mL) was stored at 4 °C in buffer A in the presence of the indicated reagents: none (○); α-ketoglutarate (10 mM) (□); EDTA (1 mM) (▲); 2-mercaptoethanol (5 mM) (△); 2-mercaptoethanol (5 mM) + EDTA (1 mM) (×); α-ketoglutarate (10 mM) + 2-mercaptoethanol (5 mM) (◐); α-ketoglutarate (10 mM) + EDTA (1 mM) (■); α-ketoglutarate (10 mM) + EDTA (1 mM) + 2-mercaptoethanol (5 mM) (●). Aliquots were withdrawn at various time intervals and assayed for enzyme activity.

This band was further checked by activity staining using the transferase assay procedure as given under Materials and Methods. Gel electrophoresis in the presence of sodium dodecyl sulfate also yielded a single band (Figure 3B). The bands observed at the top of the gel and at the bottom (position of the tracking dye marked with copper wire) appeared even when the gels were run in the absence of a protein. The Ouchterlony double-immunodiffusion test also gave a single white precipitin line confirming the homogeneity of the preparation.

Stability of Purified Glutamine Synthetase in Glycerol. Purified glutamine synthetase was quite unstable. Storage in buffer B at room temperature, 4 °C, or in the frozen state resulted in a total loss of activity in 24 h. However, in the presence of 25% (v/v) to 50% (v/v) glycerol it could be stored at 4 °C and lower temperatures for more than 2 months with 80–90% retention of activity. 25% (v/v) glycerol was chosen as 50% (v/v) glycerol made the enzyme solution extremely viscous and caused difficulty in handling. Glycerol (25% v/v) has also been found to stabilize enzymes like the dinitrophenol-stimulated adenosinetriphosphatases (ATPases) of beef

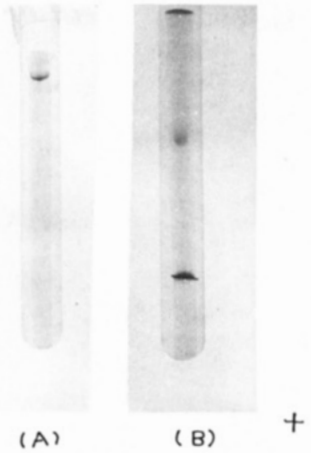

FIGURE 3: (A) Polyacrylamide disc gel electrophoresis of glutamine synthetase under nondenaturing conditions. A 32-μg aliquot of purified glutamine synthetase was subjected to gel electrophoresis in Tris–glycine buffer, pH 8.3. A current of 3 mA/tube was applied to each tube. The bands were visualized by staining with Coomassie Brilliant Blue R-250 and were destained with 7.0% acetic acid. (B) SDS–polyacrylamide gel electrophoresis of purified glutamine synthetase. Glutamine synthetase (protein: 0.4 mg/mL) was electrophoresed on 10% polyacrylamide gel in the presence of 0.1% SDS. A current of 1 mA/tube was initially applied until the protein entered the gel, and then the current was raised to 3 mA/tube for 8 h until the tracking dye reached the bottom of the gel. The gels were stained and destained as described under Materials and Methods.

heart mitochondria and of yeast (Racker et al., 1963) and 17β-hydroxysteroid dehydrogenase (Jarabak et al., 1966). The mechanism of the stabilizing effect of glycerol and related polyhydric compounds is still obscure. One suggestion that has been advanced to account for the protective effect of various organic solvents is that they may have the common property of stabilizing networks of "structured" water molecules (i.e., water–glycerol structures) which are essential for the maintenance of proper spatial configuration of the protein in the native state (Jarabak et al., 1966).

Absence of Adenylylation/Deadenylylation in Purified C. pasteurianum Glutamine Synthetase. The addition of $MgCl_2$ inhibits transferase activity of only the adenylated form of the enzyme in *Escherichia coli*, and therefore, the extent of this inhibition is used to estimate the degree of adenylylation of the enzyme (Stadtman et al., 1969). In the case of purified glutamine synthetase from *C. pasteurianum* addition of 30, 60, and 120 mM $MgCl_2$ to the γ-glutamyltransferase assay mixture containing 0.3 mM $MnCl_2$ caused 46%, 60%, and 75% inhibition, respectively, indicating partial adenylylation of the enzyme. However, when the purified enzyme (protein: 0.16 mg/mL, 0.3 mL) was treated with snake venom phosphodiesterase (protein: 0.05 mg) in 50 mM Tris-HCl, pH 7.2, and again assayed in the presence of the same Mg^{2+} concentrations, the level of activity in each case was such as to show that there was no reversal of inhibition of transferase activity. Thus, preliminary investigations indicate that the inhibition of transferase activity by Mg^{2+} is due to factors other than adenylylation or the phosphodiesterase fails to hydrolyze the adenylylated enzyme as is the case with adenylylated glutamine synthetase in *Chlorobium vibrioforme* f. *thiosulfatophilum* (Khanna & Nicholas, 1983a,b). Thus, glutamine synthetase in *C. pasteurianum* resembles either *C. vibrioforme* or other Gram-positive bacteria, where the adenylylation/deadenylylation system probably does not occur.

Requirement for Enzyme Activity. No synthetase activity was observed when any of the components glutamate, ATP, $MgCl_2$, or NH_2OH was omitted from the reaction mixture.

In the case of γ-glutamyltransferase activity, negligible activity was observed in the absence of glutamine, arsenate, $MnCl_2$, or NH_2OH. In the absence of ADP, 27% activity was observed. Glutamine synthetase of *Anabaena cylindrica* (Sawhney & Nicholas, 1978) and *Beta vulgaris* seedlings (Nesselhut & Harnischfeger, 1981) showed 65% and 45% of the activity in the complete assay mixture, respectively, in the absence of ADP. This activity could be due to ADP tightly bound to enzyme molecule. The purified preparation was free from glutaminase activity as no activity was observed when Mn^{2+} or arsenate was omitted from the transferase reaction mixture.

Effect of Molarity of Tris Buffer on Glutamine Synthetase Activity. When the enzyme was assayed in Tris-HCl buffer, pH 7.2, of different molarities, it was observed that the synthetase activity was not affected by the molarity of the buffer, while the γ-glutamyltransferase activity decreased with increase in molarity (data not shown). Maximum γ-glutamyltransferase activity was obtained in 20 mM Tris-HCl buffer, pH 7.2. Therefore, 20 mM Tris-HCl, pH 7.2, was chosen for assaying the enzyme.

Molecular Weight. The molecular weight of the purified glutamine synthetase was determined to be 1.05×10^6 by Sepharose 4B chromatography. (However, this value is only tentative as the heaviest protein marker used is thyroglobulin whose molecular weight is only 669 000.) The molecular weight seems to be unusually high compared to, for example, glutamine synthetase from *E. coli* (Woolfolk et al., 1966), *A. vinelandii* (Kleinschmidt & Kleiner, 1978), *Methylococcus capsulatus* (Murrell & Dalton, 1983), *Bacillus* species (Deuel et al., 1970; Wedler & Hoffman, 1974; Wedler et al., 1980; Donohue & Bernlohr, 1981), *Nostoc* species (Sampaio et al., 1979), *Rhodopseudomonas sphaeroides* (Engelhardt & Klemme, 1982), and the blue-green algae *Anabaena* (Sampaio et al., 1979; Stacey et al., 1977); these have molecular weights ranging between 600 000 and 700 000. However, the molecular weight of mammalian brain glutamine synthetase (Denman & Wedler, 1984) depended upon Mn^{2+} and protein concentrations, and it could have a molecular weight as high as 1×10^6 and above. The molecular weight of *C. pasteurianum* glutamine synthetase is, however, comparable to that found for glutamate dehydrogenases from a thermophilic bacillus (Epstein & Grossowicz, 1975) and beef liver (Sund & Burchard, 1968), i.e., 2×10^6, and that of rabbit skeletal muscle phosphorylase *b* kinase, which is 1×10^6 (DeLange et al., 1968).

When the purified glutamine synthetase was denatured in the presence of SDS and subjected to gel electrophoresis, only one band was noticed on Coomassie Brilliant Blue staining (Figure 3B), showing that glutamine synthetase is composed of identical subunits. The molecular weight of this band, as calculated from the standard curve, was approximately 50 000. The subunit molecular weight in this case has been found to be the same as that found for the subunits of enzyme from *Bacillus* species (Deuel et al., 1970; Wedler & Hoffmann, 1974; Wedler et al., 1980; Donohue & Bernlohr, 1981), *A. cylindrica* (Sampaio et al., 1979; Stacey et al., 1977), *Nostoc* species (Sampaio et al., 1979), *E. coli* (Woolfolk et al., 1966), *R. sphaeroides* (Engelhardt & Klemme, 1982), *Phaseolus aureus* seedlings (Seethalakshmi & Appaji Rao, 1979), and *Chlorella* (Rasulov et al., 1977) but is smaller than that of subunits of enzyme from *M. capsulatus* (Murrell & Dalton, 1983) and *Pseudomonas* species (Meyer & Stadtman, 1981). In their case, the molecular weight of the subunit was around 60 000.

Table II: Effect of Substrates on the Fluorescence of Glutamine Synthetase[a]

additions	fluorescence intensity	residual intensity (%)
enzyme alone	0.23	100
Mg^{2+}	0.22	95.6
glutamate	0.23	100
ATP	0.11	47.8
ATP + Mg^{2+}	0.10	43.4
glutamate + ATP + Mg^{2+}	0.12	52.1
NH_2OH	0.23	100
glutamine	0.23	100
Mn^{2+}	0.21	91.3
ADP	0.16	69.5
ADP + Mn^{2+}	0.17	74.0
ADP + Mn^{2+} + glutamine	0.18	78.2

[a] The experimental cuvette contained enzyme (protein: 0.11 mg) and the various substrates in 100 mM Tris-HCl, pH 7.2, at the concentrations that were used in the standard assay mixture. The excitation wavelength was set at 280 nm, and the effect on fluorescence intensity was observed at the emission maximum of 380 nm. The reference cuvette contained only buffer.

All glutamine synthetases heretofore purified have been found to consist of identical subunits. The subunit molecular weight of *C. pasteurianum* indicates that, against a molecular weight of 1×10^6, the enzyme appears to be composed of 20 identical subunits. The numbers of subunits in glutamine synthetases from various sources are usually in the range of 6 in *Chlorella* (Rasulov et al., 1977), 8 in eukaryotes (Meister, 1974) [except in the case of *Saccharomyces cerevisiae* (Hachimori et al., 1974)] and *M. capsulatus* (Murrell & Dalton, 1983), which had 10 subunits, and 12 in the case of *E. coli* (Woolfolk et al., 1966), *Bacillus* species (Deuel et al., 1970; Wedler & Hoffman, 1974; Wedler et al., 1980; Donohue & Bernlohr, 1981), *A. cylindrica* (Sampaio et al., 1979; Stacey et al., 1977), *A. vinelandii* (Kleinschmidt & Kleiner, 1978; Siedel & Shelton, 1979), and *Rhizobium japonicum* (strains CC705 and CC723) (Bhandari et al., 1983). However, approximately 16 subunits have been reported in the case of *P. aureus* seedlings (Seethalakshmi & Appaji Rao, 1979) enzyme.

Ultraviolet and Fluorescence Spectra. Glutamine synthetase absorbs maximally at 280 nm, with a trough near 260 nm and no peaks in the visible region of the spectrum, thus showing the absence of FAD, FMN, and cytochromes. Glutamine synthetases from *Bacillus subtilis* (Deuel et al., 1970), *Bacillus stearothermophilus* (Wedler et al., 1974), *Bacillus licheniformis* (Donohue & Bernlohr, 1981), *Neurospora crassa* (Lin & Kapoor, 1978), *S. cerevisiae* (Hachimori et al., 1974), *E. coli* (Woolfolk et al., 1966), and *R. sphaeroides* (Engelhardt & Klemme, 1982) also absorb maximally at 280 nm, but the A_{280}/A_{260} ratio may vary depending on whether the enzymes contain covalently bound nucleotide or not. In the case of *E. coli*, A_{280}/A_{260} is 1.3 for the adenylylated enzyme and 1.9 for the deadenylylated enzyme, and in *Bacillus* species and *N. crassa*, the value is in the range of 1.6–1.7 indicating the probable absence of an adenylyl group. Glutamine synthetase from *C. pasteurianum* has A_{280}/A_{260} ratio of 1.5. So it is uncertain from the UV absorption spectrum alone whether the enzyme is adenylylated or not, but other experiments (discussed earlier) indicated the absence of adenylylation.

The enzyme showed a fluorescence emission maximum at 380 nm when excited at 280 nm. Fluorescence studies (Table II) indicate that the ATP in the presence and absence of Mg^{2+} decreases fluorescence intensity and hence brings about conformational changes in the enzyme, while glutamate as such or in the presence of ATP + Mg^{2+} does not influence the fluorescence intensity. ADP as such and in the presence of Mn^{2+} also decreases fluorescence intensity and hence changes the enzyme conformation. Glutamine like glutamate fails to influence the fluorescence intensity. Thus preliminary investigations indicate that the *C. pasteurianum* enzyme does not behave like that of *E. coli* (Chock et al., 1974) on substrate binding and offer an interesting proposition for investigating its reaction mechanism.

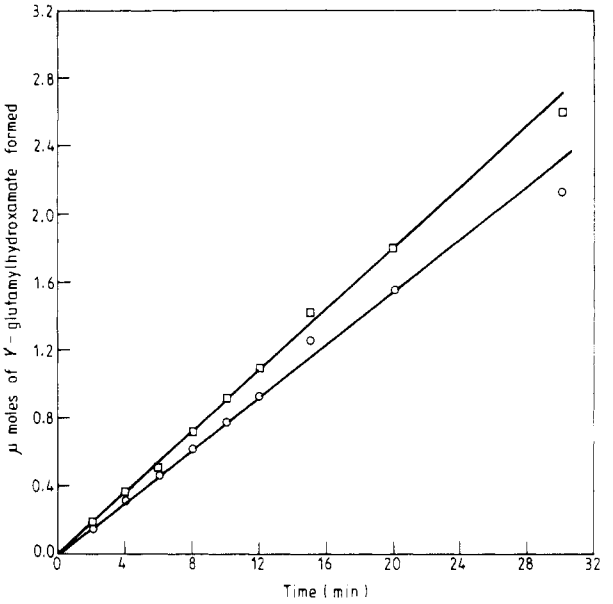

FIGURE 4: Time course of synthetase and γ-glutamyltransferase reactions. Purified glutamine synthetase was incubated with the standard assay mixture. Aliquots were withdrawn at regular time intervals and assayed for synthetase (protein: 0.09 mg/mL; 0.2 mL; 100 mM Tris-HCl buffer, pH 7.2; temperature: 50 °C) (○) and for γ-glutamyltransferase (protein: 0.09 mg/mL; 0.05 mL; 100 mM Tris-HCl buffer, pH 7.2; temperature: 30 °C) (□).

Effect of Enzyme Concentration and Time Course of the Glutamine Synthetase Reaction. The synthetase and γ-glutamyltransferase reactions show linearity up to 44 and 20 μg of protein, respectively, under our assay procedure. The formation of γ-glutamyl hydroxamate is linear upto 20 min for both the synthetase and γ-glutamyltransferase activities (Figure 4).

Optimum pH. The γ-glutamyltransferase activity has a relatively sharp pH optimum at pH 7.2, and the synthetase activity has a broader pH profile with optimal activity at pH 7.2 (Figure 5). Similarly, the enzyme from *M. capsulatus* (Murrell & Dalton, 1983) shows optimal γ-glutamyltransferase and synthetase activities at pH 7.15.

Optimum Temperature. The enzyme shows optimal synthetase and γ-glutamyltransferase activities at 50 °C (Figure 6). Glutamine synthetase from *P. aureus* seedlings (Seethalakshmi & Appaji Rao, 1979) has optimum temperature for its activity at 45 °C, while the two forms of glutamine synthetase (GS_1 and GS_2) from etiolated soybean hypocotyl (Stasiewicz & Dunham, 1979) have optimum temperatures of 50 and 45 °C, respectively. Glutamine synthetase from *Micrococcus glutamicus* (Tachiki et al., 1981) has an optimum temperature of 50 °C for the synthesizing reaction, similar to that of *C. pasteurianum*.

Energy of Activation. The energies of activation of glutamine synthetase as determined from an Arrhenius plot were 7.3 kcal/mol for the γ-glutamyltransferase activity and 13.7 kcal/mol for the synthetase activity, and the plots are linear and monophasic, thus indicating no change in enzyme conformation with temperature at which activity has been studied.

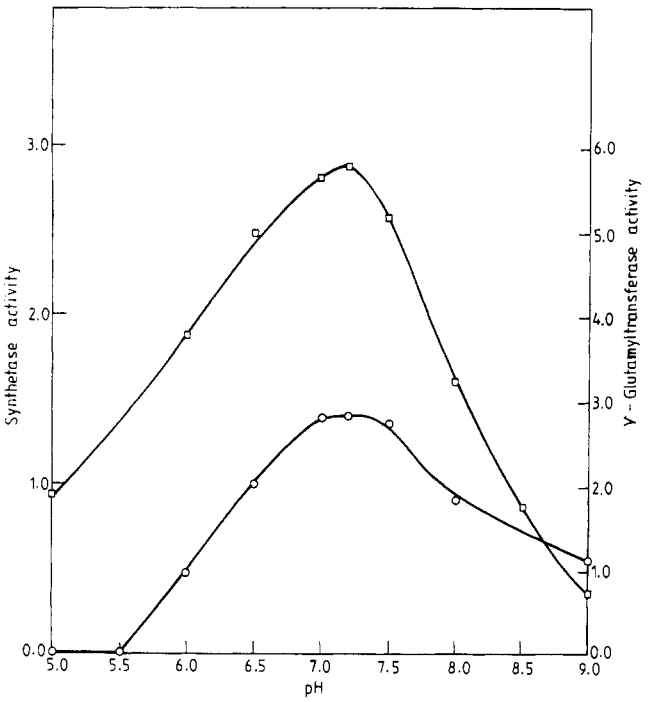

FIGURE 5: Effect of pH on glutamine synthetase and γ-glutamyltransferase activities. Glutamine synthetase was assayed for synthetase (protein: 0.15 mg/mL; 0.2 mL) (○) and γ-glutamyltransferase (protein: 0.15 mg/mL; 0.05 mL) (□) activities at different pHs (5.0–9.0) under standard assay conditions.

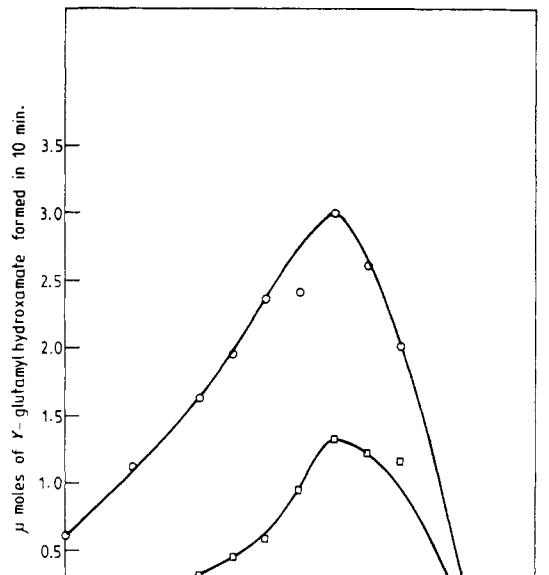

FIGURE 6: Effect of temperature on synthetase and γ-glutamyltransferase activities. Glutamine synthetase was assayed for synthetase (protein: 0.15 mg/mL; 0.2 mL) (□) and γ-glutamyltransferase (○) (protein: 0.15 mg/mL; 0.05 mL) activities under standard assay conditions at different temperatures in 100 mM Tris-HCl, pH 7.2.

Half-Life and Thermal Stability of Glutamine Synthetase. Glutamine synthetase (protein: 0.16 mg/mL) was incubated at 50 °C. Aliquots were withdrawn at different time intervals, immediately cooled in an ice bath, and then assayed for synthetase and γ-glutamyltransferase activities. The enzyme has a half-life of 8 min as measured by the synthetase and γ-glutamyltransferase activities. The ratio of the synthetase activity to the γ-glutamyltransferase activity remained unchanged at different stages of denaturation, thus again indicating that the two activities reside in the same protein.

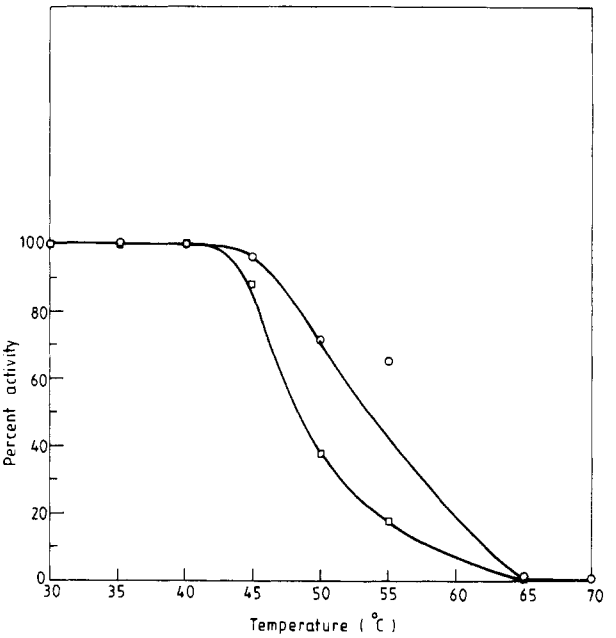

FIGURE 7: Thermal stability of glutamine synthetase. Glutamine synthetase samples (protein: 0.16 mg/mL) that were freshly purified (□) and stored in 25% (v/v) glycerol (○) were incubated at different temperatures for 10 min, cooled immediately in an ice bath, and assayed for enzyme activity under standard assay conditions. Activity of the enzyme kept at 30 °C was taken as control.

Table III: Effect of Substrates/Products on the Thermal Stability of Synthetase and γ-Glutamyltransferase Activities[a]

	relative act. (%)	
treatment	synthetase act.	γ-glutamyl-transferase act.
control	100	100
enzyme alone	38	32.7
L-glutamate	98	100
$MgCl_2$	76	87
ATP	4	5
ATP + $MgCl_2$	84	97
NH_2OH	6	0
L-glutamine	20	11
$MnCl_2$		77
ADP	6	31
$MnCl_2$ + ADP		77
sodium arsenate	32	32.7

[a] Glutamine synthetase (protein: 0.16 mg/mL) in 100 mM Tris-HCl, pH 7.2, was preincubated with the indicated concentrations of the following compounds (in mM), alone or in different combinations: L-glutamate, 90, $MgCl_2 \cdot 6H_2O$, 12; ATP, 4; NH_2OH, 60; L-glutamine, 30; ADP, 0.4; $MnCl_2 \cdot 4H_2O$, 3; sodium arsenate, 20. Each reaction mixture was incubated at 50 °C for 10 min, cooled in an ice bath, and assayed for synthetase and γ-glutamyltransferase activities under standard assay conditions. Controls with similar additions were maintained at room temperature, and their activity was taken as 100.

When the enzyme solution was incubated at different temperatures for 10 min, cooled in an ice bath, and assayed for enzyme activity, the enzyme starts losing activity at 45 °C, and only 38% activity was left at 50 °C (Figure 7). Thus, half-life as well as thermal stability studies show that the enzyme is quite unstable at 50 °C and above. But the enzyme, as discussed earlier, showed optimum activity at 50 °C, which was rather unusual. This suggested that the enzyme was being stabilized by one of the substrates/products as such or in combination. Hence the enzyme solution was preincubated with one of the substrates/products individually and in combination, incubated at 50 °C for 10 min, cooled immediately

Table IV: Metal Ion Requirement of Synthetase and γ-Glutamyltransferase Activities[a]

	act. (%)	
metal ion	synthetase act.	γ-glutamyl-transferase act.
Mg^{2+}	100	6.1
Mn^{2+}	4.3	100
Co^{2+}	17.5	15.3
Cu^{2+}	0	0
Fe^{2+}	8.7	0
Zn^{2+}		1.3
Ni^{2+}	0	0
Ca^{2+}	4.3	0

[a] Purified enzyme was exhaustively dialyzed against 100 mM Tris-HCl, pH 7.2 Aliquots (0.1 mL; protein: 0.16 mg/mL) were incubated with the metal ions at concentrations similar to those used in the respective reaction mixtures and then assayed for synthetase and γ-glutamyltransferase activities under standard assay conditions.

in an ice bath, and assayed for synthetase and γ-glutamyltransferase activities (Table III).

L-Glutamate, Mg^{2+}, and ATP + Mg^{2+} stabilized both the synthetase and γ-glutamyltransferase activities significantly, while preincubation of the enzyme with ATP, NH_2OH, L-glutamine, ADP, or sodium arsenate made the enzyme more heat-sensitive. Mn^{2+} and ADP + Mn^{2+} stabilized the γ-glutamyltransferase activity; as the presence of Mn^{2+} inhibited the synthetase activity, its stability could not be compared.

In the case of the *A. cylindrica* (Sawhney & Nicholas, 1978) enzyme, glutamine, arsenate, and ADP stabilize both the biosynthetic and γ-glutamyltransferase activities; glutamate, ATP, Mg^{2+}, and Mg^{2+} plus ATP stabilize the biosynthetic activity and Mn^{2+} and Mn^{2+} plus ADP only the transferase activity. NH_2OH inactivated the latter activity. Now, comparing these results, it is observed in *C. pasteurianum* that glutamine, ADP, ATP, and arsenate have the opposite effect while Mn^{2+}, Mn^{2+} plus ADP, and NH_2OH have a similar effect on the stability of the enzyme from the two sources. Apple (Kang & Titus, 1981) glutamine synthetase was protected against thermal inactivation by a combination of Mg^{2+} and ATP but not by either Mg^{2+} or ATP alone.

Glutamine synthetase stored in 25% (v/v) glycerol for 1 month had 70% residual activity when incubated at 50 °C compared to 38% for freshly purified enzyme when similarly treated. Thus there is an increase in thermal stability on storage in glycerol.

Metal Ion Requirement of Glutamine Synthetase. The synthetase and γ-glutamyltransferase activities were determined in the presence of the metal ions at indicated concentrations as shown in Table IV. It was observed that glutamine synthetase required Mg^{2+} in the synthesizing reaction and Mn^{2+} for the transferring reaction. Similar metal ion specificity has been observed in glutamine synthetases from *M. capsulatus* (Murrell & Dalton, 1983), *M. glutamicus* (Tachiki et al., 1981), *R. japonicum* (strains CC723 and CC705) (Bhandari et al., 1983), and *Anabaena* sp. CA (Stacey et al., 1979).

Effect of [Mg^{2+}]:[ATP] Ratio on Glutamine Synthetase Activity. The synthetase activity is a function of the Mg^{2+} and ATP concentrations, as shown in Figure 8. When the concentration of Mg^{2+} was increased in the presence of fixed levels of ATP, the synthetase activity increased to a maximum and then attained a constant value at ATP concentrations of 4 and 6 mM, but declined in the case of 12 mM ATP. In all cases, maximum activity was obtained when the [Mg^{2+}]:[ATP] ratio was 2.

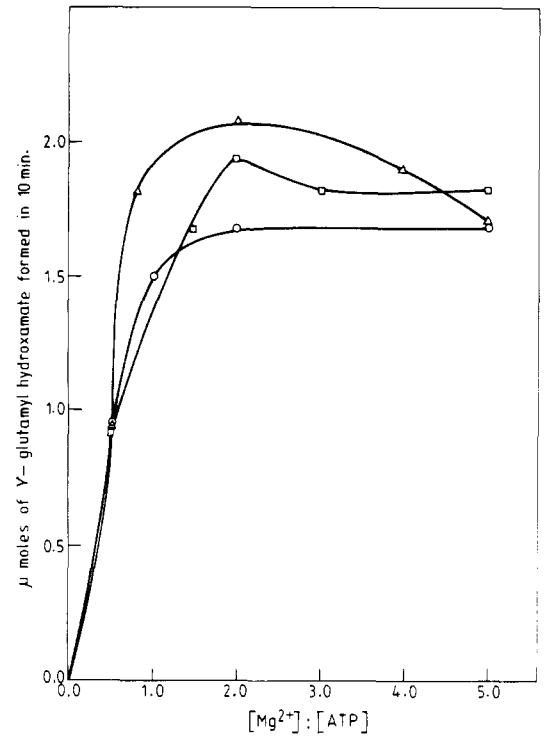

FIGURE 8: Effect of [Mg^{2+}]:[ATP] ratio on synthetase activity. Synthetase activity was determined under standard assay conditions (protein: 0.16 mg/mL) in the presence of different concentrations of $MgCl_2$ (0–60 mM) and at varying concentrations of ATP: 4 (○), 6 (□), and 12 mM (△). Synthetase activity was plotted against [Mg^{2+}]:[ATP] ratio.

Table V: Substrate/Nucleotide Specificity of Glutamine Synthetase[a]

synthetase act.		γ-glutamyltransferase act.	
substrate	act. (%)	substrate	act. (%)
L-glutamate[b]	100	L-glutamine[d]	100
D-glutamate[b]	9	L-asparagine[d]	0
L-aspartate[b]	0	albizziin[d]	9.5
ATP[c]	100	diazo-5-oxo-L-norleucine[d]	16
GTP[c]	0		
ITP[c]	16	L-azaserine[d]	21
CTP[c]	0	L-methionine sulfoximine[d]	0
UTP[c]	6	*N*,*N*'-diethylglutamine (25 mM)[d]	3.4
ADP[c]	0		
		N-methylglutamine[d]	0
		ADP[e]	100
		GDP[e]	0
		arsenate[f]	100
		phosphate[f]	64

[a] Glutamine synthetase was dialyzed against 100 mM Tris-HCl, pH 7.2. The enzyme was assayed for synthetase (protein: 0.12 mg/mL; 0.1 mL) and γ-glutamyltransferase (protein: 0.12 mg/mL; 0.05 mL) activities. The assays were carried out under standard assay conditions as given under Materials and Methods. In footnotes *b–f*, controls had their natural substrates, and their activity was taken as 100. [b] L-Glutamate was substituted by its analogues. [c] ATP was substituted by other nucleotides. [d] L-Glutamine was substituted by its analogues. [e] ADP was replaced by GDP. [f] Arsenate was substituted by phosphate.

Substrate Specificity of Glutamine Synthetase. Substrate specificity of the enzyme was determined by assaying the enzyme in the presence of its natural substrates and then in the presence of their respective analogues, keeping the rest of the assay the same. Results are shown in Table V.

The enzyme specifically synthesizes γ-glutamyl hydroxamate from NH_2OH and L-glutamate. D-Glutamate could substitute for L-glutamate with only 9% reactivity. This is similar to glutamine synthetases from *E. coli* (Woolfolk et al., 1966), *Bacillus caldolyticus* (Wedler et al., 1980), *B. stearothermophilus* (Wedler & Hoffmann, 1974), and *M. glu-*

Table VI: Apparent K_m Values of Glutamine Synthetase

substrate	K_m^{app} (mM)
synthetase reaction	
L-glutamate	3.7, 22.2
ATP	0.4
NH_2OH	0.34
γ-glutamyltransferase reaction	
L-glutamine	14
ADP	3.8×10^{-4}
NH_2OH	4.1
arsenate	2.5

tamicus (Tachiki et al., 1981), which are specific for L-glutamate, and at variance with glutamine synthetase from *S. cerevisiae* (Hachimori et al., 1974), which utilizes D-glutamate fairly well.

In the case of γ-glutamyltransferase activity, though the highest activity was obtained with L-glutamine, some of its analogues could substitute with varying activity. The absence of activity with *N,N'*-diethylglutamine or *N*-methylglutamine shows that an unsubstituted amino group is essential for catalysis.

ATP and ADP were the best nucleotide phosphates for the synthetase and transferase reactions, respectively. ITP and CTP replaced ATP with 16% and 6% reactivity, respectively. Pea seed (Pushkin et al., 1974) glutamine synthetase is also specific for ATP, and no other nucleotide triphosphate can replace it. In contrast to this, *S. cerevisiae* (Hachimori et al., 1974), *E. coli* (Woolfolk et al., 1966), and *B. stearothermophilus* (Wedler & Hoffmann, 1974) glutamine synthetases utilize other nucleotide triphosphates, particularly UTP, and show varying activity. The enzymes from *M. glutamicus* (Tachiki et al., 1981) and *E. coli* (Woolfolk et al., 1966) show maximum transferase activity with ADP.

Apparent Michaelis–Menten Constants (K_m^{app}). For determination of the apparent Michaelis–Menten constant of the enzyme for the substrates of the synthetase and γ-glutamyltransferase activities, respectively, the concentrations of two substrates were kept saturating while the concentration of the third was varied, and the enzyme activity was determined. A Lineweaver–Burk plot ($1/v$ vs. $1/s$) was drawn in each case. Linear plots were obtained in the case of ATP, NH_2OH, L-glutamine, arsenate, and ADP. The apparent K_m values are shown in Table VI. The K_m^{app} value for ATP (0.4 mM) is comparable to that found in the case of apple enzyme (0.27 mM) but much lower than that normally observed in most cases, e.g., in *B. stearothermophilus* (2.0 mM) (Wedler & Hoffmann, 1974), *B. caldolyticus* (1.2 mM for GS_I and 0.7 mM for GS_{II}) (Wedler et al., 1980), rice (1.2 mM) (Hirel & Gadal, 1980), *P. aureus* seedlings (1 mM) (Seethalakshmi & Appaji Rao, 1979), and *Aspergillus niger* (1.5 mM) (Punekar et al., 1984).

The K_m^{app} for NH_2OH (0.34 mM) for the synthetase reaction is comparable to those found in *B. stearothermophilus* (Wedler & Hoffmann, 1974), *B. caldolyticus* (GS_I and GS_{II}) (Wedler et al., 1980), *P. aureus* seedlings (Seethalakshmi & Appaji Rao, 1979), and *A. niger* (Punekar et al., 1984), which are in the range of 0.5–0.7 mM.

The K_m^{app} for glutamine (14 mM) for the γ-glutamyltransferase reaction is quite high but is comparable to those of GS_2 of etiolated soybean hypocotyl (17 mM) (Stasiewicz & Dunham, 1979) and *C. vibrioforme* f. *thiosulfatophilum* (14.7 mM) (Khanna & Nicholas, 1983a,b).

The *C. pasteurianum* enzyme has the lowest K_m^{app} for ADP (3.8×10^{-4} mM) reported so far from any other source. K_m^{app} in the case of glutamine synthetases from *E. coli* (Woolfolk et al., 1966), *Pseudomonas fluorescens* (Meyer & Stadtman, 1981), and *M. capsulatus* (Murrell & Dalton, 1983) has the values 4×10^{-2} mM, 4×10^{-3} mM, and 0.15 mM, respectively.

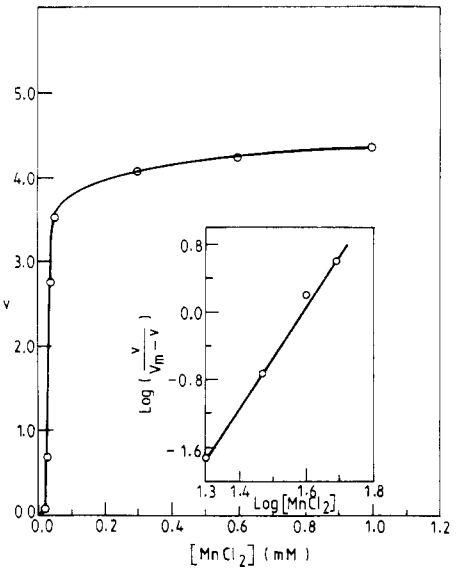

FIGURE 9: Effect of $MnCl_2$ on γ-glutamyltransferase activity. γ-Glutamyltransferase activity (protein: 0.15 mg/mL; 0.05 mL) was determined in the presence of different concentrations of $MnCl_2$ (0–1.0 mM) under standard assay conditions. The inset shows the Hill plot.

The K_m^{app} for NH_2OH (4.1 mM) in the γ-glutamyltransferase reaction is in the same range as those found for the *M. glutamicus* (Tachiki et al., 1981), *C. vibrioforme* f. *thiosulfatophilum* (Khanna & Nicholas, 1983a,b), *A. cylindrica* (Sawhney & Nicholas, 1978), *B. vulgaris* seedling (Nesselhut & Harnischfeger, 1981) etiolated soybean hypocotyl (Stasiewicz & Dunham, 1979), *E. coli* (Woolfolk et al., 1966), and *A. niger* (Punekar et al., 1984) enzymes, which have K_m^{app} in the range of 3–6 mM. The v vs. s curve for $MnCl_2$ was sigmoidal with a Hill coefficient of 6.0 (Figure 9).

While the K_m^{app} of glutamate for synthetase activity was being determined, the concentration of ATP and NH_2OH were kept at saturating values. The enzyme failed to reach the maximum velocity up to a 150 mM glutamate concentration. Concentrations higher than this could not be reached due to solubility limits. Plotting of $1/v$ vs. $1/s$ according to the method of Lineweaver and Burk gave a biphasic curve (Figure 10). At higher substrate concentration, the curve had a higher slop (K_m^{app} = 22.2 mM against 3.8 mM for lower concentration), thus indicating substrate activation. Thus the enzyme is probably allosterically regulated and is activated by glutamate. The Hill coefficient as obtained from the Hill plot (inset in Figure 10) was found to be 0.69, which is less than 1.0. This shows that glutamine synthetase shows negative cooperativity in glutamate binding. In such cases, sequential binding of ligands produces new binding sites with low affinity, which renders the enzyme less sensitive to change in substrate concentration. A similar phenomenon of negative cooperativity has been observed in the case of beef liver glutamate dehydrogenase (Olson & Anfinsen, 1953), human heart lactate dehydrogenase (Nisselbaum & Bodansky, 1961), deoxythymidine kinase (Okazaki & Kornberg, 1964), and D-glyceraldehyde-3-phosphate dehydrogenase (Conway & Koshland, 1968).

Effect of Amino Acids on Glutamine Synthetase Activity. Glutamine donates its amide nitrogen to various amino acids (Stadtman, 1973), and therefore, to check whether the enzyme activity is regulated by feedback/product inhibition or not, synthetase and transferase activities were determined in the

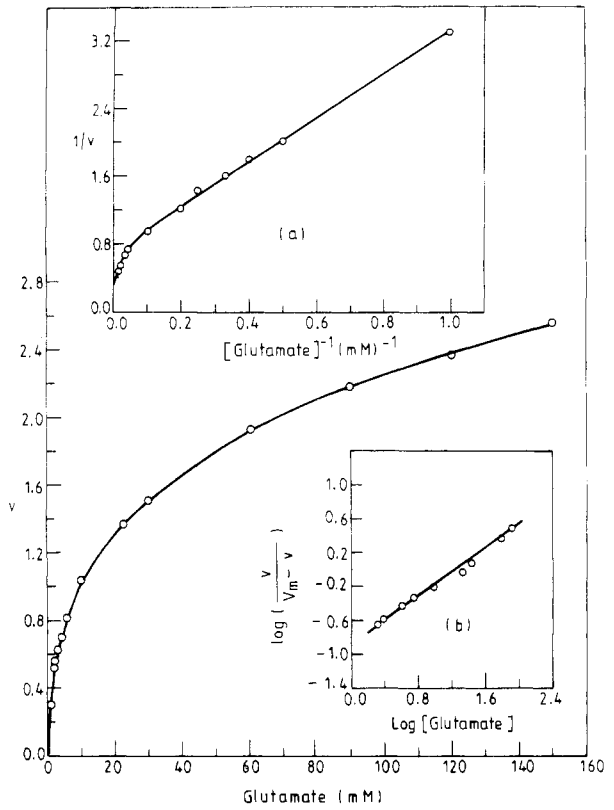

FIGURE 10: Effect of L-glutamate on glutamine synthetase activity. Synthetase activity (protein: 0.16 mg/mL; 0.1 mL) was determined in the presence of different concentrations of L-glutamate (0–150 mM) under standard assay conditions. Insets a and b show the Lineweaver–Burk plot and the Hill plot, respectively.

Table VII: Effect of Some Amino Acids on Synthetase and γ-Glutamyltransferase Activities[a]

	5 mM		20 mM	
additions	synthetase act.	γ-glutamyl-transferase act.	synthetase act.	γ-glutamyl-transferase act.
none	100	100	100	100
glycine	53.5	47.0	28.5	11.7
L-glutamate		88.2		82.3
L-glutamine	87.5		78.5	
L-alanine	50.0	55.0	32.0	28.0
L-serine	39.2	58.8	12.5	20.0
L-histidine	96.4	100	71.4	100
L-aspartic acid	66.0	82.3	50.0	50.5
L-asparagine	89.2	94.1	94.6	89.4
L-arginine	89.2	83.5	80.0	75.2
L-methionine	85.7	100	89.2	82.3
L-valine	85.7	94.7	85.7	88.2
L-tryptophan	92.0	84.6	84.0	80.0

[a] Glutamine synthetase (protein: 0.16 mg/mL) was assayed for synthetase and γ-glutamyltransferase activities under standard assay conditions in the presence of the indicated concentrations (5 and 20 mM) of amino acids.

presence of different amino acids (Table VII). Both the activities were significantly inhibited by L-serine, glycine, L-alanine, and L-aspartic acid. No inhibition was observed with L-glutamine, L-glutamate, L-histidine, L-asparagine, L-arginine, L-methionine, L-valine, and L-tryptophan.

Inhibition of glutamine synthetase by alanine and glycine has also been observed in a variety of other organisms. The enzyme from *A. vinelandii* was inhibited by L-aspartic acid besides L-alanine and glycine. Lack of inhibition by glutamine, histidine, and tryptophan was also observed in the case of this enzyme from *Anabaena* sp. CA (Stacey et al., 1979) and *A. vinelandii* (Lepo et al., 1982).

Alanine, glycine, and serine are indirectly derived from glutamine via the GS/GOGAT and transaminase reactions. Although glutamate is also a product of glutamine metabolism, it fails to show feedback inhibition, probably because it is also a substrate for the glutamine synthetase reaction. Since alanine and glycine are in dynamic equilibrium with glutamate via the transaminase reaction, it may be the function of these amino acids to sense fluctuations in the glutamine pool and inhibit glutamine synthetase accordingly.

Registry No. 5′-ATP, 56-65-5; 5′-ADP, 58-64-0; NH_2OH, 7803-49-8; Mg, 7439-95-4; Mn, 7439-96-5; L-glutamic acid, 56-86-0; L-glutamine, 56-85-9; arsenate, 15584-04-0; glycerol, 56-81-5; glutamine synthetase, 9023-70-5; γ-glutamyltransferase, 9046-27-9.

REFERENCES

Bhandari, B., Vairinhos, F., & Nicholas, D. J. D. (1983) *Arch. Microbiol. 136*, 84.

Boyer, P. D., Mills, R. C., & Fromm, H. J. (1959) *Arch. Biochem. Biophys. 81*, 249.

Chock, P. B., Rhee, S. G., & Timmons, R. B. (1974) *Enzymes (3rd Ed.) 10*, 760.

Conway, A., & Koshland, D. E., Jr. (1968) *Biochemistry 7*, 4011.

Dainty, R. H. (1972) *Biochem. J. 126*, 1055.

Dainty, R. H., & Peel, J. L. (1970) *Biochem. J. 117*, 573.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404.

DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A., & Krebs, E. G. (1968) *J. Biol. Chem. 243*, 2200.

Denman, R. B., & Wedler, F. C. (1984) *Arch. Biochem. Biophys. 232*, 427.

Deuel, T. F., Ginsburg, A., Yeh, J., Shelton, E., & Stadtman, E. R. (1970) *J. Biol. Chem. 245*, 5195.

Donohue, T. J., & Bernlohr, R. W. (1981) *J. Bacteriol. 147*, 589.

Engelhardt, H., & Klemme, J. (1982) *Arch. Microbiol. 133*, 202.

Epstein, I., & Grossowicz, N. (1975) *J. Bacteriol. 122*, 1257.

Hachimori, A., Itoh, M., & Samejima, T. (1974) *J. Biochem. (Tokyo) 76*, 1075.

Hirel, B., & Gadal, P. (1980) *Plant Physiol. 66*, 619.

Hoare, D. S., Hoare, S. L., & Moore, R. B. (1967) *J. Gen. Microbiol. 49*, 351.

Hubbard, J. S., & Stadtman, E. R. (1967) *J. Bacteriol. 93*, 1045.

Jarabak, J., Seeds, A. E., Jr., & Talalay, P. (1966) *Biochemistry 5*, 1269.

Kang, S., & Titus, J. S. (1981) *Physiol. Plant. 53*, 239.

Khanna, S., & Nicholas, D. J. D. (1983a) *Arch. Microbiol. 134*, 98.

Khanna, S., & Nicholas, D. J. D. (1983b) *FEMS Microbiol. Lett. 18*, 173.

Kleiner, D. (1975) *Arch. Microbiol. 104*, 163.

Kleiner, D. (1979) *Arch. Microbiol. 120*, 263.

Kleinschmidt, J. A., & Kleiner, D. (1978) *Eur. J. Biochem. 89*, 51.

Lepo, J. E., Stacey, G., Wyss, O., & Tabita, F. R. (1979) *Biochim. Biophys. Acta 568*, 428.

Lepo, J. E., Wyss, O., & Tabita, F. R. (1982) *Biochim. Biophys. Acta 704*, 414.

Lin, W. S., & Kapoor, M. (1978) *Can. J. Biochem. 56*, 927.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.

Meister, A. (1974) *Enzymes (3rd Ed.) 10*, 699.

Meyer, J. M., & Stadtman, E. R. (1981) *J. Bacteriol. 146*, 705.

Murrell, J. C., & Dalton, H. (1983) *J. Gen. Microbiol. 129*, 1187.
Nesselhut, T., & Harnischfeger, G. (1981) *Physiol. Plant. 51*, 329.
Nisselbaum, J. S., & Bodansky, O. (1961) *J. Biol. Chem. 236*, 323.
Okazaki, R., & Kornberg, A. (1964) *J. Biol. Chem. 239*, 275.
Olson, J. A., & Anfinsen, C. B. (1953) *J. Biol. Chem. 202*, 841.
Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand. 26*, 507.
Punekar, N. S., Vaidyanathan, C. S., & Appaji Rao, N. (1984) *J. Biosci. 6*, 17.
Pushkin, A. V., Evstigneeva, Z. G., & Kretovich, V. L. (1974) *Biokhimiya (Moscow) 39*, 533.
Racker, E., Pullman, M. E., Penefsky, H. S., & Silverman, M. (1963) *Proc. Int. Congr. Biochem., 5th, 1961 5*, 303.
Rasulov, A. S., Evstigneeva, Z. G., Kretovich, V. L., Stel'-maschuk, V. Y., Samsonidze, T. G., & Kiselev, N. A. (1977) *Biokhimiya (Moscow) 42*, 350.
Rosenblum, E. D., & Wilson, P. W. (1950) *J. Bacteriol. 59*, 83.
Sampaio, M. A. M., Rowell, P., & Stewart, W. D. P. (1979) *J. Gen. Microbiol. 111*, 181.
Sawhney, S. K., & Nicholas, D. J. D. (1978) *Planta 139*, 289.
Seethalakshmi, S., & Appaji Rao, N. (1979) *J. Biosci. 1*, 13.
Siedel, J., & Shelton, E. (1979) *Arch. Biochem. Biophys. 192*, 214.
Stacey, G., Tabita, F. R., & Van Baalen, C. (1977) *J. Bacteriol. 132*, 596.
Stacey, G., Van Baalen, C., & Tabita, F. R. (1979) *Arch. Biochem. Biophys. 194*, 457.
Stadtman, E. R. (1973) in *The Enzymes of Glutamine Metabolism* (Pruisner, S., & Stadtman, E. R., Eds.) p 1, Academic Press, New York.
Stadtman, E. R., Ginsburg, A, Ciardi, J. E., Yeh, J., Hennig, S. B., & Shapiro, B. M. (1969) *Adv. Enzyme Regul. 8*, 99.
Stasiewicz, S., & Dunham, V. L. (1979) *Biochem. Biophys. Res. Commun. 87*, 627.
Sund, H., & Burchard, W. (1968) *Eur. J. Biochem. 6*, 202.
Tachiki, T., Wakisaka, S., Kumagai, H., & Tochikura, T. (1981) *Agric. Biol. Chem. 45*, 1487.
Umbarger, H. E. (1969) *Annu. Rev. Biochem. 38*, 323.
Warburg, O., & Christian, W. (1941) *Biochem. Z. 310*, 384.
Weber, K., & Osborn, M. (1975) *Proteins (3rd Ed.) 1*, 179.
Wedler, F. C., & Hoffmann, F. M. (1974) *Biochemistry 13*, 3207.
Wedler, F. C., Shreve, D. S., Kenney, R. M., Ashour, A. E., Carfi, J., & Rhee, S. J. (1980) *J. Biol. Chem. 255*, 9507.
Wilson, G. S., & Miles, A. (1975) in *Topley and Wilsons Principles of Bacteriology, Virology and Immunity*, Vol. 1, p 1129, Arnold, London.
Woolfolk, C. A., Shapiro, B. M., & Stadtman, E. R. (1966) *Arch. Biochem. Biophys. 116*, 177.
Zelitch, J., Rosenblum, E. D., Burris, R. H., & Wilson, P. W. (1951) *J. Biol. Chem. 191*, 295.

The Sulfhydryls of Firefly Luciferase Are Not Essential for Activity†

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ABSTRACT: Firefly luciferase, containing an average of seven free sulfhydryls per two 50 000-dalton polypeptides, was modified by various sulfhydryl reagents. The differential reactivities of the sulfhydryls in luciferase protected by substrates allow one to define three categories of these groups: Class SH-III contains three sulfhydryls that are not involved in enzymatic activity. Class SH-II contains two sulfhydryls whose modification by different reagents causes varying effects on activity ranging from 0 to 60% inactivation. These sulfhydryls are not essential but may be important structurally or sterically. Class SH-I contains two sulfhydryls that are protected by substrates, either dehydroluciferyl adenylate or dehydroluciferin alone, and are located at or near the active site. The SH-I sulfhydryls are vicinal in the enzyme as demonstrated by their ability to form a disulfide bond. They have also been shown to exist on a single polypeptide chain. Modification of the SH-I groups by most reagents results in complete loss of enzymatic activity; reaction with methyl methanethiosulfonate produces an enzyme that emits only red light whereas native luciferase emits yellow-green light. Evidence is presented that the modified enzyme, while catalytically active, has a distorted active site. It is concluded that these two SH-I sulfhydryls are not essential for activity.

The following reactions are catalyzed by firefly luciferase:[1]

$$E + LH_2 + ATP \overset{Mg^{2+}}{\rightleftharpoons} E\text{–}LH_2AMP + PP_i \quad (1)$$

$$E\text{–}LH_2AMP + O_2 \rightarrow E\text{–}P + AMP + CO_2 + h\nu \quad (2)$$

$$E + L + ATP \overset{Mg^{2+}}{\rightleftharpoons} E\text{–}LAMP + PP_i \quad (3)$$

Reaction 1 is an activation step resulting in the formation of the enzyme-bound adenylate of luciferin. Reaction 2 is the oxidative and light-emitting step. The product oxyluciferin

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[1] Abbreviations: LH_2, luciferin; L, dehydroluciferin; LAMP, dehydroluciferyl adenylate; pMB, *p*-mercuribenzoate; MMTS, methyl methanethiosulfonate; NEM, *N*-ethylmaleimide; IAAm, iodoacetamide; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoate); TNB, 5-thio-2-nitrobenzoate; SDS, sodium dodecyl sulfate; diamide, 1,1′-azobis(*N*,*N*-dimethylformamide); TEMED, tetramethylethylenediamine; EDTA, ethylenediaminetetraacetic acid.

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