JOURNAL OF BACTERIOLOGY, Feb. 1989, p. 1158–1165
0021-9193/89/021158-08$02.00/0

Vol. 171, No. 2

Purification and Properties of Glutamine Synthetase from the Non-N_2-Fixing Cyanobacterium *Phormidium laminosum*

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Received 28 July 1988/Accepted 2 November 1988

Soluble glutamine synthetase activity (L-glutamate:ammonia ligase, ADP forming, EC 6.3.1.2) was purified to electrophoretic homogeneity from the filamentous non-N_2-fixing cyanobacterium *Phormidium laminosum* (OH-1-p.Cl_1) by using conventional purification procedures in the absence of stabilizing ligands. The pure enzyme showed a specific activity of 152 μmol of γ-glutamylhydroxamate formed · min^{-1} (transferase activity), which corresponded to 4.4 μmol of P_i released · min^{-1} (biosynthetic activity). The relative molecular mass of the native enzyme was 602 kilodaltons and was composed of 12 identically sized subunits of 52 kilodaltons. Biosynthetic activity required the presence of Mg^{2+} as an essential activator, although Co^{2+} and Zn^{2+} were partially effective. The kinetics of activation by Mg^{2+}, Co^{2+}, and Zn^{2+} were sigmoidal, and concentrations required for half-maximal activity were 18 mM (h = 2.2), 6.3 mM (h = 5.6), and 6.3 mM (h = 2.45), respectively. However, transferase activity required Mn^{2+} (K_a = 3.5 μM), Cu^{2+}, Co^{2+}, or Mg^{2+} being less effective. The substrate affinities calculated for L-Glu, ammonium, ATP, L-Gln, and hydroxylamine were 15, 0.4, 1.9 (h = 0.75), 14, and 4.1 mM, respectively. Optimal pH and temperature were 7.2 and 55°C for biosynthetic activity and 7.5 and 45°C for transferase activity. The biosynthetic reaction mechanism proceeded according to an ordered three-reactant system, the binding order being ammonium, L-Glu, and ATP. The presence of Mn^{2+} or Mg^{2+} drastically affected the thermostability of transferase and biosynthetic activities. Heat inactivation of biosynthetic activity in the presence of Mn^{2+} obeyed first-order kinetics, with an E_a of 76.8 kcal (ca. 321 kJ) · mol^{-1}. Gly, L-Asp, L-Ala, L-Ser and, with lower efficiency, L-Lys and L-Met inhibited both activities of glutamine synthetase, whereas L-Arg inhibited biosynthetic activity and L-Tyr, L-Lys, and L-Glu inhibited only transferase activity. No cumulative inhibition was observed when mixtures of amino acids were used. Biosynthetic activity was inhibited by AMP (K_i = 7 mM), ADP (K_i = 2.3 mM), *p*-hydroxymercuribenzoate (K_i = 25 μM), and L-methionine-D,L-sulfoximine (K_i = 2 μM). The enzyme was not activated in vitro by chemically reduced *Anabaena* thioredoxin. This is the first report of glutamine synthetase activity purified from a filamentous non-N_2-fixing cyanobacterium.

Glutamine synthetase (GS; L-glutamate:ammonia ligase, ADP forming, EC 6.3.1.2), in collaboration with glutamate synthase (EC 1.4.7.1), plays a central role in ammonium assimilation in N_2-fixing heterotrophic bacteria (20), photosynthetic bacteria (3), and a wide range of cyanobacteria (see reference 11 for a recent review). However, in addition to the GS-glutamate synthase cycle, glutamate dehydrogenase (EC 1.4.1.4) (16) and alanine dehydrogenase (EC 1.4.1.1) (24) may also contribute to ammonium assimilation in cyanobacteria, especially under certain nutritional conditions of nitrogen availability. Recently, an alternative route to GS-glutamate synthase for the primary assimilation of ammonium in *Anabaena* sp. strain 1F, involving the participation of GS in collaboration with carbamylphosphate synthetase (EC 2.7.2.9), has been proposed (5). Consequently, GS is subject to intensive regulatory control.

Although the GS isolated from bacterial sources are similar in a number of features, such as molecular size (about 600 kilodaltons [kDa]), subunit structure (dodecamers composed of identical subunits of about 50 kDa), and the requirement of divalent cations for activity, their regulatory properties appear to be quite different. For example, the enzyme from *Escherichia coli* (28) and several other gram-negative bacteria (13) is regulated by covalent modification through adenylylation-deadenylylation. In contrast, the GS from gram-positive bacteria is susceptible to feedback regulation by intracellular concentrations of adenine nucleotides, amino acids, and divalent cations rather than by covalent modification (17). However, in blue-green algae (cyanobacteria), the enzyme can be activated by dithiol compounds in vitro or by reduced thioredoxin (27). The cyanobacterial enzyme is not susceptible to regulation by covalent modification, and the availability of divalent cations and presence of feedback inhibitors (mainly Gly, L-Ala, and L-Ser) may play the dominant role in regulating GS in vivo (22, 30). Moreover, the cyanobacterial enzyme shows cumulative inhibition with mixtures of inhibitors (i.e., AMP, L-Ser, and L-Asp), which indicates that there are independent binding sites on the enzyme for the inhibitors (30). The *Anabaena flos-aquae* GS was also found to be regulated by the energy charge of the cell, and cooperative inhibition was caused by CTP and any single nucleotide (18).

GS from several species of the filamentous N_2-fixing cyanobacterial genera *Anabaena* (18, 23, 25, 29) and Nostoc (25) has been purified to electrophoretical homogeneity and characterized. Despite abundant information on the enzyme from N_2-fixing cyanobacteria, only data regarding the GS from the unicellular non-N_2-fixing cyanobacterium *Anacystis nidulans* (9, 10) are available to date. In this nonfilamentous organism, the enzyme appears to be membrane bound (9, 10), and the use of detergents is required to solubilize the enzyme.

In this paper, we describe the complete purification of the soluble GS from the filamentous non-N_2-fixing cyanobacte-

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rium *Phormidium laminosum*, using conventional methods in the absence of any added stabilizing ligand, which was reported to be essential for purification of the cyanobacterial enzyme. We also discuss some relevant physical and kinetic properties of the *P. laminosum* enzyme as compared with other cyanobacterial enzymes and report data on the reaction mechanism, effect of Mg^{2+} and Mn^{2+} on the thermal stability of the enzyme, and the effect of some nucleotides, L-amino acids, and inhibitors.

MATERIALS AND METHODS

Chemicals. L-Amino acids, nucleotides, inhibitors, γ-glutamylhydroxamate, bovine serum albumin, acrylamide, *N,N'*-methylenebisacrylamide, Coomassie brilliant blue R-250, and subunit molecular weight markers (Dalton Mark VII) were from Sigma Chemical Co. (St. Louis, Mo.). Gel filtration standard proteins were from Boehringer GmbH (Mannheim, Federal Republic of Germany). Sephacryl S-300 was from Pharmacia Biotechnology (Uppsala, Sweden). DEAE-cellulose (DE-52) was from Whatman Biochemicals (Maidstone, United Kingdom). Coomassie brilliant blue G-250, inorganic salts (chlorides), and other biochemicals were obtained from E. Merck AG (Darmstadt, Federal Republic of Germany).

Organisms and growth conditions. *P. laminosum* OH-1-p.Cl_1 was originally obtained from R. W. Castenholz (University of Oregon, Eugene). Cells were grown photoautotrophically in pure culture at 45°C in 10-liter Pyrex carboys (Corning Glass Works, Corning, N.Y.) containing 6 liters of mineral medium D (4) supplemented with $NaCO_3H$ (0.5 g · $liter^{-1}$). Cultures were stirred with an air stream bubbled (about 3 liters · min^{-1}) under continuous illumination provided by white fluorescent lamps with a photon flux density at the surface of the vessels of about 100 μE · m^{-2} · s^{-1}.

Cells from 3- to 4-day-old cultures were harvested by centrifugation at 9,000 × *g* for 20 min, washed twice with cold 50 mM Tris hydrochloride (pH 8.0) (buffer A), resuspended in the same buffer, and stored at −20°C until used.

Enzyme assays. Both transferase and biosynthetic activities of GS were determined for 15 min at 35°C as described by Shapiro and Stadtman (28), except that Tris hydrochloride (pH 7.2) (buffer B) replaced the original imidazole buffer. Transferase activity was followed by measuring the formation of γ-glutamylhydroxamate in the reaction mixture (final volume, 2 ml), which contained (in micromoles): buffer B, 80; L-Gln, 60; $MnCl_2$, 6; ADP, 0.8; hydroxylamine (neutralized with NaOH), 120; Na_2HAsO_4, 37.5; and an appropriate amount of enzyme. The reaction was started by adding arsenate, which was omitted in the control tubes. One unit of enzyme catalyzed the formation of 1 μmol of γ-glutamylhydroxamate · min^{-1}.

Biosynthetic activity was assayed in reaction mixtures (final volume, 0.2 ml) containing (in micromoles): buffer B, 10; L-Glu, 10; NH_4Cl, 10; $MgCl_2$, 13; ATP, 1.5; and an appropriate amount of enzyme. The reaction was initiated by adding ATP, which was omitted in the control tubes. One unit of enzyme catalyzed the release of 1 μmol of P_i · min^{-1}.

Purification of GS. All steps were carried out at 0 to 4°C in buffer A. Cells (about 0.28 g of chlorophyll *a*) were thawed and sonicated for 15 min in an ice bath. The preparation was centrifuged at 40,000 × *g* for 30 min, and the supernatant was fractionated with solid $(NH_4)_2SO_4$. The 45 to 70% saturation fraction was redissolved in buffer A and extensively dialyzed against the same buffer. The dialysate was applied to a DEAE-cellulose (DE-52) column (2.2 by 30 cm) equilibrated in buffer A. After being washed with 200 ml of 0.2 M KCl containing buffer A, the enzyme was eluted with buffer A containing 0.4 M KCl. Active fractions were combined, concentrated, and filtered through a Sephacryl S-300 column (3.2 by 30 cm) equilibrated and developed with buffer A containing 50 mM KCl. The most active fractions were collected, combined, and applied to a second DE-52 column (1.6 by 30 cm) equilibrated with buffer A. After the column was washed with 250 ml of 0.2 M KCl containing buffer A, the enzyme was eluted with a 600-ml linear gradient of KCl from 0.2 to 0.4 M. Fractions containing pure GS (as judged by polyacrylamide gel electrophoresis) were pooled, concentrated by ultrafiltration (Amicon Diaflo PM-30 membrane), and stored at −20°C. The pure enzyme was stable for several months when stored at this temperature.

Analytical methods. Protein was determined by the method of Bradford (2), using crystalline bovine serum albumin as the standard. Chlorophyll *a* was estimated spectrophotometrically after extraction into acetone (1).

Polyacrylamide gel disc electrophoresis. Enzyme purification was followed by analytical polyacrylamide gel electrophoresis according to the method of Davis (6) with 5% (wt/vol) acrylamide gels. The protein bands were stained at 60°C for 30 min with 0.05% (wt/vol) Coomassie brilliant blue R-250 and destained by diffusion.

Transferase activity was located in gels according to the method of Lepo et al. (15). Biosynthetic activity was located by incubating the gel in the standard reaction mixture. After 1 h of reaction, the gel was transferred to buffer B containing 50 mM $CaCl_2$ and incubated at room temperature until a stable band of insoluble $CaHPO_4$ appeared.

Subunit composition of pure GS was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, performed in vertical slabs (15 by 12 cm) by a modification of the method described by Laemmli (14) in 10% (wt/vol) acrylamide gels. Gels were run at room temperature for 30 min at 25 V and then at 70 V until the bromophenol blue reached the bottom of the gel. Bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.2 kDa) were used as protein standards.

Determination of molecular mass. The apparent molecular mass of the enzyme was estimated by molecular sieve chromatography on a Sephacryl S-300 column (1.6 by 92 cm; void volume, 56 ml) calibrated with ferritin (660 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and cytochrome *c* (12.4 kDa) in buffer A containing 1 mM 2-mercaptoethanol and 50 mM KCl.

RESULTS

Cell rupture and enzyme solubility. Cells were disrupted by a number of methods. Sonicated preparations showed the maximal activity, although 86% of that could be measured in the lysozyme-treated cell preparation (30), which indicated that GS was easily extractable under mild homogenization conditions and was soluble. After centrifugation for 1 h at 40,000 × *g*, more than 80% of activity found in the original sonic extract could be recovered in the 40,000 × *g* supernatant, and an increase in specific activity of up to 1.5-fold could be obtained upon centrifugation of the crude preparation.

Enzyme purification. A complete purification protocol for *P. laminosum* GS which yielded 2.5 mg of pure enzyme is

TABLE 1. Purification of GS from *P. laminosum*[a]

Step	Total protein (mg)	Total activity (U)	Sp act (U · mg of protein^{-1})	Purification (fold)	Yield (%)
40,000 × *g* supernatant	382.8	812	2.12	1.0	100
45–70% $(NH_4)_2SO_4$	109.7	501	4.56	2.1	62
DE-52 I eluate	25.4	459	18.1	8.5	56
Sephacryl S-300 eluate	3.9	404	103.7	49.5	50
DE-52 II eluate	2.5	380	152.0	71.7	47

[a] The enzyme was purified from 16 liters of cells (17.4 μg of chlorophyll · ml^{-1}). Activity was measured by the transferase assay. The transferase activity-to-biosynthetic activity ratio was about 35 in all purification steps.

summarized in Table 1. The enzyme was routinely purified more than 70-fold with a recovery of 45 to 60% and showed a specific transferase activity of 152 U · mg of protein^{-1}, which corresponded to a biosynthetic activity of 4.4 U · mg of protein^{-1}.

After the second ion-exchange chromatographic step, a single band of protein (Fig. 1) coinciding with both the transferase and biosynthetic activity bands was detected by nondenaturing polyacrylamide gel electrophoresis, which suggested that the enzyme was homogeneous.

Molecular mass and subunit composition. The relative molecular mass (M_r) of the native enzyme determined by molecular sieve chromatography was 602 kDa (data not shown). When the pure enzyme was incubated at 100°C for 2 min with 3% (wt/vol) sodium dodecyl sulfate and 5% (vol/vol) 2-mercaptoethanol, subsequent electrophoresis in detergent-equilibrated polyacrylamide gels revealed the presence of a single band of protein with an M_r of about 52 kDa. This result, together with the relative molecular mass of the native enzyme, suggests that *P. laminosum* GS consists of 12 identically sized subunits.

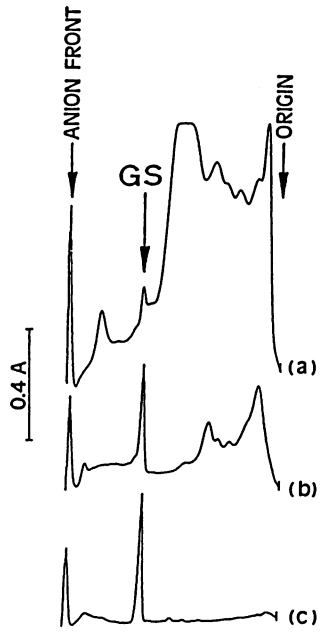

FIG. 1. Densitograms of *P. laminosum* GS in polyacrylamide gels at different purification stages: 40,000 × *g* supernatant (A), Sephacryl S-300 eluate (B), and DE-52 II eluate (C). After staining for protein as indicated in Materials and Methods, gels were scanned at 600 nm with a Shimadzu CS-930 scanner.

TABLE 2. Substrate affinities of different cyanobacterial GS

Cyanobacterium[a]	K_m or $[S]_{0.5}$ (mM)[b]				
	ATP	L-Glu	NH_4^+	NH_2OH	L-Gln
N_2 fixing					
Anabaena sp. strain CA (30)	NR[c]	5	0.2	NR	NR
Anabaena sp. strain 7120 (22)	0.32	2.1	0.02	NR	NR
Anabaena cylindrica (25)	NR	2	1	NR	NR
Anabaena flos-aquae (18)	NR	NR	NR	NR	10
Non-N_2 fixing					
Anacystis nidulans (10)	0.7	5	0.02	NR	NR
Phormidium laminosum	1.9	14.8	0.4	4.1	13.9

[a] References for the enzyme purified from other cyanobacteria are given in parentheses for comparison.

[b] Michaelis-Menten kinetics were obtained except in the case of ATP for the *P. laminosum* enzyme, in which negative cooperativity (h = 0.75) could be detected.

[c] NR, Not reported.

Urea caused reversible dissociation of the native enzyme into inactive subunits. Therefore, when the pure enzyme was incubated at 35°C for 30 min with urea and then assayed by the standard method in the presence of the same concentration of urea, both biosynthetic and transferase activities decreased progressively; 50% of the initial activity was measured in equal portions of enzyme treated and assayed with 3.75 M urea. No activity could be recovered after enzyme treatment with urea at 6.5 M or higher. The effect caused by urea could be almost completely reversed by dialyzing the enzyme.

Kinetic properties. In Tris hydrochloride buffer, the optimal pHs for transferase and biosynthetic activities of the *P. laminosum* GS were 7.5 and 7.2, respectively.

In Table 2, substrate affinities of the pure enzyme are summarized and compared with those of the enzyme purified from other cyanobacteria. With ATP, the *P. laminosum* enzyme showed nonhyperbolic kinetics denoting negative cooperativity (h = 0.75), whereas hyperbolic (Michaelis-Menten type) kinetics were obtained for the other enzymes, for which K_m values were computer calculated from direct linear plots (8).

To investigate the reaction mechanism of the biosynthetic activity catalyzed by GS, the pure enzyme was assayed at a fixed concentration of one of its substrates, the concentrations of the other two being varied. For each fixed substrate, data were analyzed by using double-reciprocal plots of velocity versus the concentration of one of the variable substrates in the left-hand plot and versus that of the other variable substrate in the right-hand plot. Thus, at fixed 7.5 mM ATP (Fig. 2A) and plotting $1/v$ versus 1/[L-Glu], the set of lines obtained for various concentrations of NH_4^+ intercepted at a common point on the $1/v$ axis, indicating that NH_4^+ and L-Glu bind to the enzyme in an ordered way. When these data for $1/v$ were replotted versus $1/[NH_4^+]$ (Fig. 2B), the new set of lines obtained intercepted at a common point in the second quadrant, indicating that NH_4^+ binds to the enzyme before L-Glu. The iteration of this analysis (Fig. 2C through F) indicated that the binding order of substrates to GS was NH_4^+, L-Glu, ATP.

Like the GS purified from other sources, the *P. laminosum* enzyme required the presence of a divalent cation as an essential activator. Maximal biosynthetic activity required about 65 mM Mg^{2+} (100% activity), although Co^{2+} (36% activity) and Zn^{2+} (6% activity) at the same concentration were partially effective. Concentrations of Mg^{2+} higher than

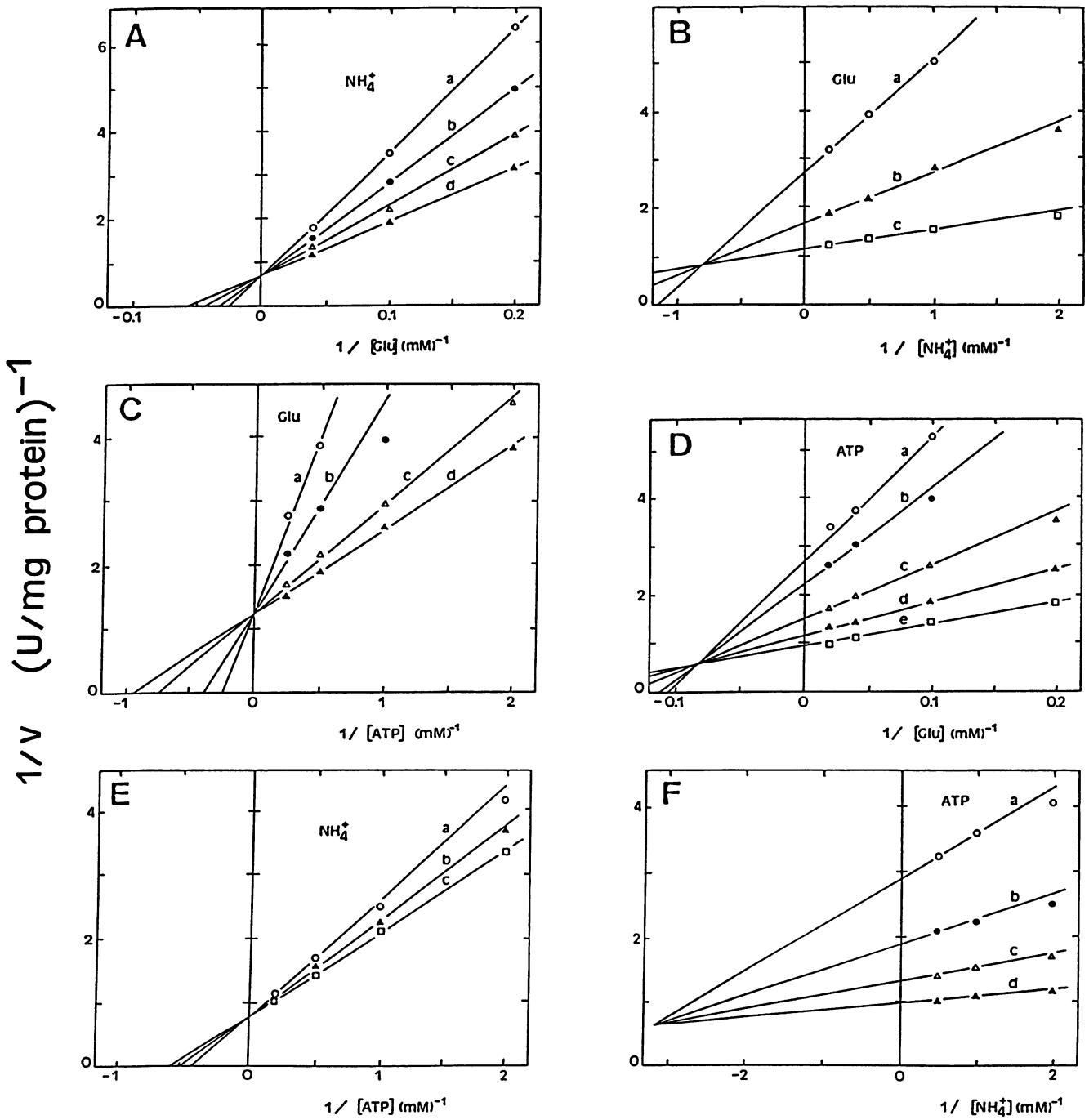

FIG. 2. Kinetic mechanism of biosynthetic activity catalyzed by the GS from *P. laminosum*. The pure enzyme (4.5 μg of protein) was assayed by using a fixed concentration of one substrate and various concentrations of the other substrates. The fixed substrates were 7.5 mM ATP (A and B), 50 mM NH_4^+ (C and D), and 50 mM L-Glu (E and F). Data were analyzed by using Lineweaver-Burk plots. Data shown in panels A, C, and E were replotted after exchanging the axis of the variable substrates (B, D, and F, respectively).

80 mM were less effective. However, a much lower concentration of Mn^{2+} (about 20 μM) was required for maximal transferase activity. At 3 mM metal cation, Cu^{2+} (52% activity), Co^{2+} (44% activity), Mg^{2+} (25% activity), and Fe^{2+} (6% activity) could partially replace Mn^{2+} (100% activity). Other divalent cations, such Ca^{2+}, Hg^{2+}, and Ni^{2+}, were completely ineffective for both activities. The curves obtained when biosynthetic activity was plotted against Mg^{2+}, Co^{2+}, or Zn^{2+} concentrations were sigmoidal. When these kinetic data were analyzed by using Hill plots, positive cooperativity ($h > 1$) was detected in all cases and the calculated divalent cation concentrations required for half-maximal activity were 18 mM ($h = 2.2$), 6.3 mM ($h = 5.6$), and 6.3 mM ($h = 2.45$) for Mg^{2+}, Co^{2+}, and Zn^{2+},

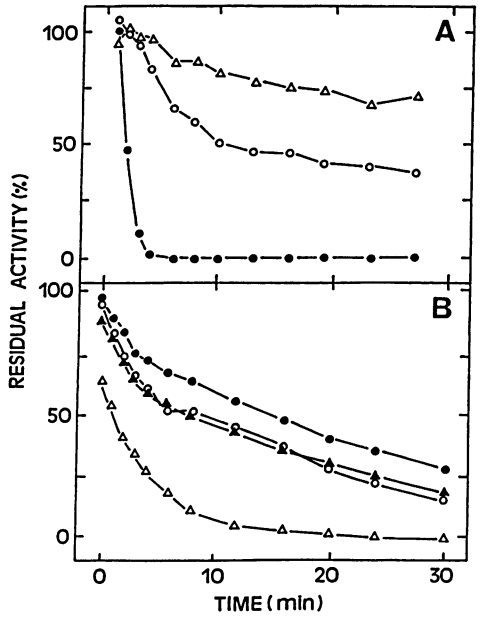

FIG. 3. Thermal denaturation of *P. laminosum* GS. The pure enzyme (0.3 mg · ml^{-1}) was incubated at 55°C (A) or 66°C (B) under an air atmosphere in sealed vials. At intervals, vials were cooled in an ice bath and the residual activity was determined by either the transferase (A) or the biosynthetic (B) assay as described in Materials and Methods. The enzyme portions had been previously dialyzed against 50 mM Tris hydrochloride buffer (pH 8.0) alone (○) or supplemented with 2 mM EDTA (●), 2 mM $MnCl_2$ (△), or 2 mM $MgCl_2$ (▲).

respectively. However, the activation kinetics caused by Mn^{2+} on transferase activity were hyperbolic, and a value of K_a = 3.5 μM was calculated.

Effect of temperature on enzyme activity and stability. Optimal temperatures for biosynthetic and transferase activities were about 55 and 45°C, respectively. After incubation of the pure enzyme for 10 min at various temperatures in the presence of the respective incomplete reaction mixture, thermal inactivation above 45°C (transferase) or 50°C (biosynthetic) was observed. Moreover, above 70°C (transferase) or 75°C (biosynthetic), total enzyme inactivation occurred (data not shown).

However, the presence or absence of either Mg^{2+} or Mn^{2+} in the incubation medium dramatically affected the thermal stability of both transferase and biosynthetic activities (Fig. 3). Therefore, the decrease in the thermal stability of biosynthetic activity caused by the presence of Mn^{2+} was studied at various temperatures (Fig. 4). A set of straight lines was obtained when the log (residual activity/initial activity) was plotted versus time (data not shown), indicating that thermal inactivation obeyed a first-order reaction. The logarithms of the first-order constants, calculated from the slopes of the lines, were plotted according to Arrhenius; from the slope of the resulting line (Fig. 4, insert), an activation energy of 76.8 kcal (ca. 331.33 kJ) · mol^{-1} was calculated for the thermal inactivation of enzyme in the presence of Mn^{2+}.

Regulatory properties. Several nucleotides (CMP, cAMP, dAMP, AMP, GMP, and ADP), the thiol reagent *p*-hydroxymercuribenzoate (*p*-HMB), and the L-Glu analog L-methionine-D,L-sulfoximine (MSX) were tested as potential effectors of the biosynthetic activity of the pure GS. At 7.5 mM nucleotide, only ADP (42% inhibition) and AMP (28% inhibition) caused a significant effect. A negligible effect (<5% of inhibition) was observed with the other nucleotides.

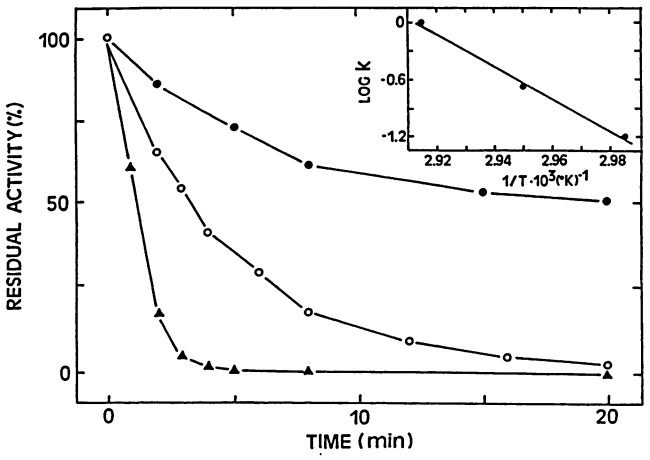

FIG. 4. Thermostability of biosynthetic activity of *P. laminosum* GS at various temperatures. The enzyme was dialyzed against 1 mM $MnCl_2$ and incubated at 62°C (●), 66°C (○), and 70°C (▲) as described in the legend to Fig. 4. Residual activity is the percentage of that shown by the nonheated enzyme, which was 0.52 U · mg of $protein^{-1}$. Insert shows the Arrhenius plot used to determine the activation energy for thermal inactivation of the enzyme, which was 76.8 kcal (ca. 321 kJ) · mol^{-1}.

Biosynthetic activity was more susceptible than transferase activity to inhibition by *p*-HMB and MSX. *p*-HMB inhibited transferase activity by 80% at 25 mM, whereas the same degree of inhibition of biosynthetic activity was achieved at 1 mM. Similarly, 1 mM MSX inhibited transferase activity by 20%, whereas 25 μM MSX inhibited biosynthetic activity by 70%.

The kinetics of inhibition caused by ADP, AMP, *p*-HMB, and MSX were studied in detail. The type of inhibition caused by these compounds with respect to L-Glu and the K_i values are summarized in Table 3.

Most L-amino acids were tested (at 5 mM) as feedback inhibitors of the pure *P. laminosum* GS. Only Gly, L-Asp, L-Ala, and L-Ser inhibited significantly both biosynthetic and transferase activities when measured under standard assay conditions. L-Cys, L-Arg, and L-Met caused about 5 to 15% inhibition, and L-Asn, L-Gln, L-His, L-Lys, L-Trp, and L-Val did not inhibit (<5% inhibition) biosynthetic activity. On the other hand, L-Cys, L-Tyr, L-Lys, L-Met, and L-Glu inhibited by about 5 to 25%, whereas L-Arg, L-Asn, L-His, L-Ile, L-Leu, L-Pro, L-Phe, L-Thr, L-Trp, and L-Val caused no inhibition of transferase activity. The kinetics of inhibition caused by the most effective amino acids were further examined (Table 4). No cumulative inhibition of biosynthetic or transferase activity was detected when mixtures (binary or ternary) of inhibitory amino acids were used.

TABLE 3. Inhibitors of biosynthetic activity of *P. laminosum* GS[a]

Inhibitor	Type	K_i (mM)
ADP	Linear mixed (α = 7.0)	2.3
AMP	Linear mixed (α = 10.5)	7.0
p-HMB	Linear mixed (α = 5.5)	0.025
MSX	Competitive pure	0.002

[a] Pure enzyme (about 2 μg of protein) was assayed under standard biosynthetic conditions at several fixed concentrations of inhibitor and various concentrations of L-Glu.

TABLE 4. Inhibition of *P. laminosum* GS by amino acids[a]

Amino acid inhibitor	Inhibition of:					
	Biosynthetic activity			Transferase activity		
	%	Type	K_i (mM)	%	Type	K_i (mM)
None	0			0		
Gly	41	Linear mixed (α = 5.4)	1.8	58	Competitive pure	0.9
L-Ser	22	Uncompetitive pure	8.0	63	Competitive pure	1.2
L-Ala	16	Competitive pure	3.9	50	Competitive pure	2.9
L-Asp	24	Competitive pure	1.8	30	ND[b]	ND

[a] Pure enzyme (about 2 μg of protein) was assayed under standard conditions at several fixed concentrations of inhibitor and various concentrations of L-Glu (biosynthetic) or L-Gln (transferase). Amino acids were used at a concentration of 0.5 mM.
[b] ND, Not determined.

It is remarkable that reduced glutathione (γ-glutamylcysteinylglycine) at 5 mM inhibited biosynthetic activity by 46%, whereas the same concentration of either L-Cys (13% inhibition) or other thiol compounds, such as 2-mercaptoethanol and dithioerythreitol (<5% inhibition in both cases), was less effective.

DISCUSSION

GS has been completely purified from a number of N_2-fixing cyanobacteria (18, 23, 25, 29) and the non-N_2-fixing *A. nidulans* (10). In Table 5, some relevant properties of the enzyme purified from various cyanobacteria are summarized for comparison. To avoid or minimize enzyme inactivation during purification, several stabilizing ligands (Table 5), such as L-Glu and Mg^{2+} (29) or EDTA, 2-mercaptoethanol, L-Glu, and Mg^{2+} (25), were routinely used.

All of the GS purified so far are soluble except the enzyme of *A. nidulans* (9, 10). In this unicellular cyanobacterium, about 50% of the total activity was found associated with the membrane fraction of cell-free preparations (9), and the use of detergents, such as dodecyldimethylamine oxide and cetyltrimethylammonium bromide (9) or alkyltrimethylammonium bromide (10), was absolutely required for enzyme solubilization.

However, GS from the filamentous non-N_2-fixing cyanobacterium *P. laminosum* was apparently soluble and could be easy and completely extracted without detergents and purified to electrophoretic homogeneity by using conventional purification procedures in the absence of any added stabilizing agent.

Sampaio et al. (25) reported that *Anabaena cylindrica* GS could be present in a fully active and a deactivated form. The native enzyme lost activity, and its dodecameric structure became disaggregated upon dialysis in the absence of stabilizing ligands. However, Florencio and Ramos (10) reported the presence in *A. nidulans* of a single active form which was independent of the presence of ligands in the dialysis buffer, although these workers used Mg^{2+} and dithioerythreitol throughout enzyme purification. Stability during purification of the *P. laminosum* enzyme was not increased in the presence of the stabilizing agents reported by different researchers. Moreover, the dialysis of enzyme against 50 mM Tris hydrochloride buffer (pH 7.2), alone or supplemented with 2 mM EDTA, apparently did not cause changes in the type of kinetics or the affinity of the enzyme for Mg^{2+} (data not shown). These results are in agreement with those reported by Florencio and Ramos (10), who concluded that regardless of whether the transition between the active and deactivated forms of GS is operative in regulation of the enzyme in N_2-fixing cyanobacteria, it seems that this mechanism is probably absent in non-N_2-fixing cyanobacteria.

The native molecular mass (about 600 kDa) and oligomeric composition (12 identically sized subunits of about 50 kDa) of the enzyme from *P. laminosum* appear to be common features in most bacteria (7, 32, 34) as well as in cyanobacteria (Table 5) except *Anabaena flos-aquae* (18), for which an M_r of 430 kDa was found. However, significant differences were reported in substrate affinities (Table 2). Thus, the K_m or $[S]_{0.5}$ values found for the *P. laminosum* enzyme were generally higher than those reported for other cyanobacterial enzymes. The enzyme showed hyperbolic kinetics for all substrates except ATP, for which a Hill coefficient (h = 0.75) indicating negative cooperativity was calculated. It is also remarkable that the affinity of the GS for ammonium

TABLE 5. Properties of GS purified from cyanobacteria

Cyanobacterium[a]	Enzyme soluble	Stabilizing agents required	Purification		Sp act (U · mg of protein^{-1})		Native M_r (kDa)	Subunit	
			Yield (%)	Fold	Transferase	Biosynthetic		M_r (kDa)	No.
N_2 fixing									
Anabaena sp. strain CA (29, 30)	Yes	Yes	17	56	78–150	2–17	590	50	12
Anabaena sp. strain 7120 (22, 23)	Yes	Yes	26	100	123	9.4	600	50	12
Anabaena cylindrica (25)	Yes	Yes	20	213	32	9.4	591–660	50	12
Anabaena flos-aquae (18)	Yes	Yes	50	380	400	8	430	NR[b]	NR
Non-N_2 fixing									
Anacystis nidulans (10)	No	Yes	57	64	205	10	575	47	12
Phormidium laminosum	Yes	No	47	72	152	4.4	602	52	12

[a] References are given in parentheses for comparison.
[b] NR, Not reported.

varied widely among the cyanobacteria and ranged from 20 μM (10, 22) to 1 mM (25). A value of 0.4 mM was calculated for the *P. laminosum* enzyme.

Like the GS characterized from other cyanobacteria (12, 26, 29), the enzyme purified from *P. laminosum* required 65 mM Mg^{2+} for maximal biosynthetic activity. The *Anabaena cylindrica* GS required 25 mM Mg^{2+} supplied alone, but Co^{2+} and Mn^{2+} each supported up to 20% of this activity (12). Moreover, Ca^{2+} and Ni^{2+} partially supported the activity of the enzyme from both *Anabaena cylindrica* (26) and *Anabaena* sp. strain CA (29). Ip et al. (12) reported that Mn^{2+} strongly inhibited the Mg^{2+}-dependent biosynthetic activity of the *Anabaena cylindrica* GS and suggested that Mn^{2+} may play a regulatory function in vivo. In contrast, Nakano and Kimura (21) reported that the activity of the *Bacillus cereus* GS was greatly enhanced by the addition of Mn^{2+} to the Mg^{2+}-dependent assay mixture; they suggested that the binding sites for both cations are separate from each other in the active site of the enzyme and that both sites must be occupied for normal activity of GS in vivo.

Merkler et al. (19) reported that bound metal ions and substrates caused dramatic changes in the temperatures at which aggregation and loss of activity occurred for the GS enzyme from the extreme thermophile *Bacillus caldolyticus*. Previously, Wedler et al. (33) reported that the native GS from ovine brain could be routinely isolated with Mn^{2+} tightly bound. If this is the case with the *P. laminosum* enzyme, these findings would help to explain the different thermal stabilities of the enzyme shown in Fig. 4, where the presence of Mn^{2+} affected the stability of both activities in opposite ways, i.e., protecting transferase activity but diminishing the thermostability of biosynthetic activity. However, when the enzyme was presumably deprived of metal cations upon dialysis against EDTA, the thermostability of transferase activity was decreased and that of biosynthetic activity was increased.

ADP and AMP acted as linear mixed inhibitors, with respect to L-Glu, of the *P. laminosum* GS (Table 3). However, both nucleotides act as noncompetitive inhibitors of the *Anabaena cylindrica* enzyme (26). Like the *A. nidulans* GS (10), the *P. laminosum* enzyme was strongly inhibited by *p*-HMB, which suggests the presence of a sulfhydryl group important for both biosynthetic and transferase activities. However, the effect of other thiol reagents, such *N*-ethylmaleimide, was much weaker (only <10% inhibition at 1 mM). At very low concentrations (<50 μM), MSX acted as a reversible competitive inhibitor, with a K_i of 2 μM, whereas, as has been found for the enzyme from other sources, inhibition was irreversible at higher MSX concentrations.

Feedback inhibition by amino acids appears to be the major regulatory mechanism of the cyanobacterial enzyme. Thus, as is the case for the enzyme from N_2-fixing cyanobacteria (18, 22, 25, 30), the biosynthetic activity of *P. laminosum* GS was inhibited mainly by Gly, L-Asp, L-Ser, and L-Ala, whereas L-Gln, L-His, and L-Trp were ineffective. However, the *A. nidulans* enzyme was also found to be inhibited by L-Ala and L-Asp, but Gly and L-Ser showed little effect (10). Consequently, regarding the regulatory effect exerted by L-amino acids, it seems apparent that the *P. laminosum* GS resembles the GS of filamentous N_2-fixing cyanobacteria rather than that of the nonfilamentous non-N_2-fixing *A. nidulans*. However, there are significant differences among the enzyme from different sources with respect to the type of inhibition caused and the calculated apparent K_i values. Thus, the inhibitory effect of L-Asp was competitive pure (K_i = 1.8 mM) for the *P. laminosum* enzyme but was noncompetitive (K_i = 10 mM) in *A. nidulans* (10), noncompetitive (K_i = 13 mM) in *Anabaena* sp. strain CA (30), noncompetitive (K_i = 9.6 mM) in *Anabaena* sp. strain 7120 (22), and of mixed type in *Anabaena cylindrica* (26). It is also worth mentioning that in *P. laminosum* GS, in contrast to the case with the enzyme from other cyanobacteria (18, 30), no cumulative effect caused by inhibitory amino acids was detected, which suggests that amino acids do not bind to the *P. laminosum* enzyme in separate or dependent sites.

It is well established that GS from blue-green algae can be activated in vitro by reduction with dithiols or more efficiently by reduced thioredoxin (27). The presence of different monothiol or dithiol compounds (at 5 mM) caused no activation of the pure *P. laminosum* GS. Moreover, dithiothreitol-reduced thioredoxin from *Anabaena variabilis* or *Anabaena cylindrica* was also ineffective. The inhibitory effect caused by reduced glutathione on the *P. laminosum* enzyme (similar to that caused by L-Ser) was probably due to the presence of the inhibitory amino acids L-Ser and L-Cys in the structure of the tripeptide.

The *P. laminosum* GS has at least two unusual characteristics in comparison with the enzyme from other cyanobacteria: no stabilizing ligands are required during purification, and neither a cumulative nor a cooperative inhibition effect was caused by mixtures of inhibitory amino acids. Despite the strong resemblances in physical structure of the procaryotic enzyme and taking into account the dissimilar regulatory properties, it is apparent that the cyanobacterial GS is more diverse than was previously thought.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Comisión Asesora de Investigación Científica y Técnica (CAICYT 928/84) and the Basque Country Government (X-86.049). A.A. and F.B. were the recipients of scholarships (PFPI) from the Spanish Ministry of Education and Science.

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