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TWO REGULATORY ISOZYMES OF GLUTAMINE SYNTHETASE FROM BACILLUS CALDOLYTICUS, AN EXTREME THERMOPHILE

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Summary:

Two glutamine synthetases (EC 6.3.1.2) have been purified to homogeneity from B. caldolyticus strain YTP, grown at 70° on a minimal, defined medium. The enzymes are virtually identical in size and molecular weight (12 subunits of MW 50,000), but differ in their isoelectric points, electrophoretic mobility, net charge, inherent thermal stability, affinity for substrates, and activity responses to metal ions and pH. Of primary interest is the observation that the more acidic form (pI = 5.2), E_I, is strongly feedback-regulated by certain amino acids derived from glutamine (Gly, L-Ala, L- and D-ser) but not by L-Glu or AMP, whereas the less acidic form (pI = 5.5), E_{II}, is inhibited most strongly by L-Gln and AMP, but not by the above amino acids. Both enzymes are inhibited strongly by ADP, CTP, NAD, glucosamine-6-P, less strongly by nucleotide diphosphates and L-Trp, and are activated by nucleotide monophosphates other than AMP. These results suggest that overall regulation of glutamine synthetase by the full spectrum of end product metabolites derived from L-Gln is accomplished by regulatory isozymes in this extremely thermophilic organism.

Introduction:

Stadtman and co-workers have shown that in microorganisms the regulation of glutamine synthetase by metabolic end products derived from glutamine is exceedingly complex (1). The amount of functional information contained as binding sites per enzyme subunit is so high that we have been led to ask how much of this could be maintained in a microorganism adapted to an extreme environment and how this is accomplished. Our previous studies with the enzyme from a moderate thermophile, B. stearothermophilus (2,3,4) at 55° showed that minimal simplifications in responses to feedback regulators was required. Thus, in extending this research to an extreme thermophile, B. caldolyticus, capable of growth at 85-90°, we have discovered isozymes that respond to different groups of feedback modifiers.

Materials and Methods:

Biochemicals were of highest purity obtainable from Sigma Chemical Company, all salts and inorganics were ACS analytical grade from Fisher

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Scientific, and all water was double-distilled from glass.

Growth of B. caldolyticus, strain YTP, from NASA Ames Labs, Moffett Field, CA, was carried out on 3% nutrient agar plates in minimal medium. The liquid minimal medium was composed of: 3.0 mM K_2HPO_4, 0.9 mM KH_2PO_4, 4.3 mM NaCl, 0.25 mM $MnCl_2 \cdot 6H_2O$ and $MgCl_2 \cdot 6H_2O$, 0.36 mM $FeSO_4 \cdot 7H_2O$, 0.034 mM $CaCl_2$, 15 mM glucose, 1.5-2.5 g/L Bacto-tryptose (Difco) or trypticase-peptone (BBL) and 0.15 ml/L trace elements solution (TES). The TES contained (per L): 2.37g $ZnCl_2$; 5.0g H_3PO_4, 0.25g $CuSO_4 \cdot 5H_2O$, 0.25g $Na_2MoO_4 \cdot 2H_2O$, 0.48g $CoCl_2 \cdot 6H_2O$ and 5.0 ml H_2SO_4. All growth was carried out at 68-70°C in volumes up to 300L.

Purification of glutamine synthetases was carried out according to the following outlined procedure:

(1) Crude extract: obtained from cells in 50 mM imidazole-acetate buffer, pH 7.0, with 10 mM $MnCl_2$ (1/3:w/v) sonicated 5 min/50 ml volume, then nucleic acids removed with 1% streptomycin sulfate.

(2) Ammonium sulfate precipitation at 60% sat., then back-extraction with 35% sat. in 20% original volume.

(3) Affinity chromatography on Bio-gel A15m (CNBr-activated, linked to ε-amino-caproic acid) coupled to L-methionine-(S,R)-sulfoximine (MSOX), eluted with 0-0.5 M gradient of KCl.

(4) Gel filtration on Bio-gel A 1.5m, then A 0.5m, with 10 mM Im-OAc buffer, pH 6.5, 5 mM $MnCl_2$.

(5) Ion exchange chromatography on DEAE-Agarose with a gradient of 0-0.5m KCl.

(6) Affinity Chromatography on either the MSOX-Agarose column of step 3, or an Agarose-Cibachrom (Affigel Blue, Bio Rad) with a 0-0.3 M or 0-1.0 M gradient of KCl, respectively. Either procedure gives pure, separated isozymes.

The overall yield is in excess of 75%, with purification of 100-200X for each enzyme, depending on cell growth conditions.

Electrophoresis procedures included (a) migration of native enzyme in 5% polyacrylamide gels with the pH 7.2 buffer system of Chrambach (5); (b) isoelctric focusing in 4% gels containing 8 M urea and 1.7% Nonidet NP-40 with pH 3-6 LKB Ampholytes; (c) SDS (0.1%)- polyacrylamide (7) gels.

Ultracentrifugation sedimentation velocity patterns were carried out with 5 mg/ml protein at pH 7.0 at 40,000 RPM in a Beckman Spinco Model E ultra-centrifuge with schlieren optics.

Assays for enzyme activity were carried out at 70°C at pH 6.50, 25 mM Im-OAc, with 50 mM L-glutamate, 10 mM $MnCl_2$-ATP, and 10 mM of either NH_4Cl or NH_2OH. The "biosynthetic" activity with NH_4Cl was measured by P_i release colorimetrically (A_{660}) by addition of $FeSO_4$-acid molybdate complex formation (2, 3, 4, 6). The γ-glutamyl-hydroxamate synthetase (GHS) activity was measured colorimetrically (A_{540}) by addition of acidic $FeCl_3$ (2, 3, 4, 6).

Results and Discussion:

Microbiology: Recent investigations reveal that Bacillus caldolyticus, strain YTP (7, 8) as isolated and supplied by NASA Ames Labs contains two variants that can be distinguished with regard to morphology of colonies, growth on various defined media, and other characteristics (9, 10). Grown on nutrient agar, one of these produces large translucent yellowish colonies, while the other grows more slowly and forms small, opaque, white colonies. Pure cultures of each were separately grown on our defined media, harvested, the cells broken, and enzyme purified through step 3 of the purification scheme. Each variant was found to contain both electrophoretically separable enzymes E_I and E_{II} (see Figure 1, top) as assayed by slicing the polyacrylamide gels into 3 mm pieces and assaying for glutamine synthetase activity. This argues that the two variant strains are very closely related and are most likely variants of the same species.

Cell growth and nutrient effects. Addition of either NH_4^+ or L-glutamine above 2 mM reduced the specific activity of total glutamine synthetase (measured by GHS assay , pH 6.5, with $MnCl_2$) to 25-30% of the original value. The molecular basis for this effect is not yet known. Enzyme activity levels were not sensitive to addition (in mM) of L-Glu (20), or L-Asp (20), nor Trypticase below 2.5g/L. Well-aerated cultures of cells in the defined media with no added NH_4Cl typically doubled their A_{650} about every 30 min at 70° up to A_{650} ~ 1.5 with a cell yield of 5g wet weight/L culture.

Criteria of homogeneity. The two enzymes purified from low-NH_4^+ grown cells differ from each other in virtually every parameter except quaternary structure, size, shape and molecular weight. The enzymes differ with respect to net change, E_I migrating faster on 5% polyacrylamide gel electrophoresis at pH 7.2 than E_{II}. Their isoelectric points were found to be 5.2 and 5.5 for E_I and E_{II}, respectively (see Materials and Methods). Our preliminary experiments with electron microscopy and sedimentation in the ultracentrifuge do not distinguish or separate the two forms, however.

Distinct differences in basic kinetic properties, substrate specificity,

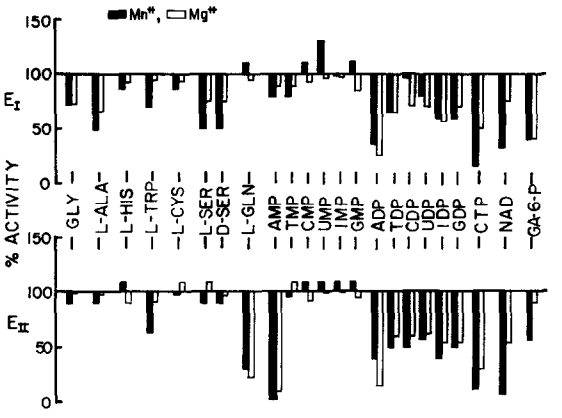

Figure 1: Inhibition of biosynthetic activity of B. caldolyticus glutamine synthetases E_I (top) and E_{II} (bottom) by feedback modifiers derived from L-Gln. Each modifier was used at 10 mM, except that L-Trp and TMP were used at 5 mM. Nucleotide di- and tri-phosphates were studied with divalent metal ion at 1 mM in excess of the total nucleotide level; (filled bars) Mn^{2+}, pH 6.20; (open bars) Mg^{2+}, pH 7.50 in the biosynthetic assay with substrate levels as indicated in the Materials and Methods section.

and responses to pH and metal ions have been found and will be recounted in detail in later publications. Whether adenylylation-deadenylylation occurs with E_I or E_{II} is not yet known definitively, despite preliminary observations (11) that suggested it may occur.

Regulation of E_I and E_{II} by metabolites. The results of screening experiments with relatively high concentrations of both substrates and added modifiers are shown in Figure 1. Striking differences are seen to occur depending on the enzyme (E_I or E_{II}) and divalent cation (Mn^{2+} or Mg^{2+}) present. Limitation of the level of each substrate, in turn, also, alters inhibitory behavior (not shown). Despite the obvious complexity of these inter-related effects, the data of Figure 1 clearly indicate that (a) E_I is inhibited significantly by Gly, L-Ala, L- and D-Ser, and L-Trp, but not by L-Gln or AMP; (b) E_{II} is not inhibited significantly by amino acids except L-Trp but strongly by L-Gln and AMP; (c) CTP, NAD, and glucosomine-6-P (GA-6-P) inhibit both enzymes rather strongly at 10 mM; (d) nucleotide monophosphates other than AMP may slightly stimulate or inhibit activity, whereas nucleotide diphosphates inhibit activity moderately. Overall, glutamine synthetase activity is seen to respond to the full spectrum of metabolic end products derived from L-Gln in the cell; however, the differences

between regulation of E_I and E_{II} in (a) and (b) above point to an isozymic "division of labor" to accomplish this goal. This strategy may result from the necessary evolution of more stable, less flexible proteins capable of survival at 70° or above. Although two enzymes in a mesophilic Rhizobium were also recently reported (12), their role in vivo is not yet known.

A complete description of the physical, kinetic, and regulatory properties of these enzymes is in the process of completion and preparation for publication.

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