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4-METHYLENEGLUTAMINE SYNTHETASE: A NEW AMIDE SYNTHETASE PRESENT IN GERMINATING PEANUTS

Harry C. Winter, Ti-Zhi Su,[1] and Eugene E. Dekker

Department of Biological Chemistry
The University of Michigan
Ann Arbor, MI 48109

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SUMMARY: Enzymatic activity which catalyzes the synthesis of 4-methyleneglutamine from 4-methyleneglutamic acid + ammonia was detected in and partially purified from cotyledons of peanut seeds germinated 5 to 7 days. This activity was separated from glutamine and asparagine synthetases by ammonium sulfate precipitation and DEAE-cellulose chromatography. The enzyme is distinct from these other amide synthetases in its substrate specificity, lack of amide/hydroxylamine exchange, and use of ammonium ion as amide donor together with formation of AMP from ATP. The activity is quite labile in solution, but is retained as a precipitate in ammonium sulfate or when frozen in 12.5% glycerol at -77°C. This activity might be responsible for catalyzing the rapid synthesis of 4-methyleneglutamine which occurs in germinating peanuts.

The non-protein amino acid, 4-Megln[2], is formed rapidly during germination of peanut seeds and becomes a major soluble nitrogen-containing compound in the mature plant wherein it is presumed to have a role in nitrogen translocation (1,2). Nothing is known regarding the biosynthesis of this amino acid, but the final step is most likely γ-amidation of 4-Meglu. We previously established that 4-Meglu is amidated by glutamine synthetase (E.C. 6.3.1.2.) present in germinating peanut seeds; the level of activity with 4-Meglu, however, is very low and the K_m so high that this reaction probably does not account for the observed rapid synthesis of 4-Megln (3). We report here the partial purification of an amide synthetase from germinating peanut cotyledons which is distinct from both glutamine and asparagine synthetases and has considerably greater specificity and affinity toward 4-Meglu than does glutamine synthetase.

[1]Permanent address: Beijing Feedstuffs Institute, Beijing, People's Republic of China.
[2]Abbreviations used: 4-Megln, 4-methyleneglutamine; 4-Meglu, 4-methyleneglutamic acid.

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We have named this activity 4-methyleneglutamine synthetase since it might well be the enzyme responsible for 4-Megln synthesis *in vivo*.

METHODS: Peanuts (Jumbo Virginia, Burpee Seed Co., Warminster, Pa., or Early Bunch Virginia, Park Seed Co., Greenville, S.C.) were germinated in trays of moist sterile sand containing Captan fungicide (Orthocide, Chevron Chem. Co.). After 5 to 7 days of germination, the cotyledons were removed and homogenized with polyvinylpyrrolidone in "extraction buffer" (0.1 M Tris·HCl, pH 8.5, in 25% glycerol containing 20 mM NaCl, 20 mM KCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 25 mM β-mercaptoethanol) in a Waring Blendor cooled to 0°C. All remaining operations were carried out at 0-4°C.

After filtration through cheesecloth and subsequent centrifugation, the crude extract was immediately brought to 0.8% protamine sulfate, stirred, and centrifuged. Enzymatic activity was precipitated from the supernatant fluid with 42% saturated $(NH_4)_2SO_4$ (258 g/l), then redissolved in "eluting buffer" (0.05 M Tris·HCl, pH 7.5, in 12.5% glycerol containing 20 mM NaCl, 20 mM KCl, 0.5 mM dithiothreitol, and 12.5 mM β-mercaptoethanol) and fractionated on a column of Bio-Gel A0.5m (5x90 cm) equilibrated with the same buffer. Enzyme activity eluted at ~950 ml; fractions containing 4-Megln synthetase activity were immediately placed on a column of DEAE-cellulose (1.5x16 cm) equilibrated with eluting buffer. After the column was washed, a linear gradient of NaCl (0.15 M/100 ml) in the same buffer was applied. 4-Megln synthetase was eluted at ~0.15 M NaCl; fractions containing the activity were pooled and brought to 60% saturated $(NH_4)_2SO_4$ (390 g/l). The precipitated enzyme was stable in this medium for at least 10 days at 4°C. For use, the precipitate was recovered by centrifugation, dissolved in 2-3 ml of eluting buffer, and desalted quickly on a small (8-ml bed volume) column of Sephadex G-25 with the same buffer. In solution, the enzyme was relatively unstable; it lost half of its activity within 24 hrs at 4°C but activity was retained for at least 10 days when the solution was quickly frozen in dry ice-ethanol and stored at -20° or -77°C.

Enzyme assays: 4-Megln synthetase, glutamine synthetase, and asparagine synthetase activities were assayed by ATP-dependent acyl hydroxamate formation from the corresponding amino acid and hydroxylamine (3). The assay for 4-Megln synthetase contained (final volume = 0.75 ml): 50 μmol Tricine, pH 7.5, 10 μmol $MgSO_4$, 50 μmol $NH_2OH·HCl$ (neutralized with KOH), 6 μmol ATP, 10 μmol phosphocreatine, 15 units creatine phosphokinase, 12.5 μmol L-4-Meglu, and enzyme solution, 0.1-0.3 ml. When assays were conducted on fractions which also contained glutamine synthetase as determined by the transferase assay (4), 15 μmol methionine sulfoximine were added to inhibit this activity (5). This precaution was necessary since 4-Meglu is a substrate (albeit a poor one) for glutamine synthetase (3). Reactions were incubated 45 min at 37°C and terminated by adding an equal volume of $FeCl_3$-stop mixture (3). One unit of enzymic activity catalyzed formation of 1 μmol/min of acyl hydroxamate.

Materials: L-4-Meglu was purified from aqueous extracts of peanut seedlings by ion exchange chromatography (details to be published). Protamine sulfate, Tris, EDTA, dithiothreitol, β-mercaptoethanol, DEAE-cellulose, Tricine, $NH_2OH·HCl$, ATP, phosphocreatine, creatine phosphokinase, and L-methionine-DL-sulfoximine were products of Sigma Chemical Co.; Bio-Gel A0.5m and polyvinylpyrrolidone (Polyclar AT) were from Bio-Rad; $(NH_4)_2SO_4$ was "ultrapure" from Schwarz-Mann. All other reagents were analytical grade from various commercial suppliers.

RESULTS AND DISCUSSION: The peak of 4-Megln synthetase activity recovered after DEAE-cellulose chromatography (Fig. 1) was enriched ~160-fold over the activity present in crude extracts, and was free of both glutamine synthetase

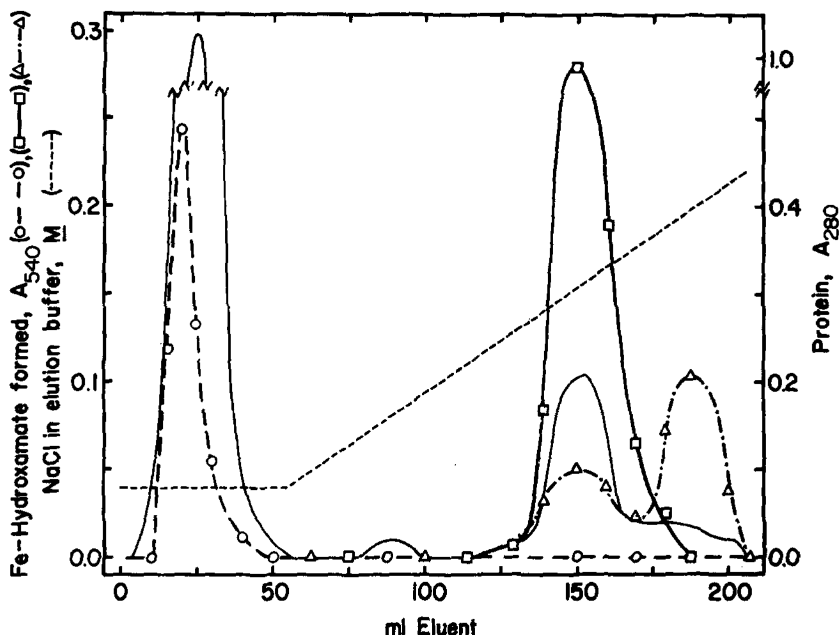

Fig. 1. Elution profiles of total protein (——), glutamine synthetase (O--O), 4-methyleneglutamine synthetase (□—□), and asparagine synthetase (Δ···Δ) activities from a column of DEAE-cellulose. Glutamine synthetase (transferase) assay was performed with 50-μl samples and a 15 min reaction time; 4-Megln and asparagine synthetase (acyl hydroxamate synthesis) with 300-μl samples and a 45 min reaction time.

and the major peak of asparagine synthetase activities. Best preparations so far obtained had a specific activity = 45 munits/mg and a turnover number of 12.7 (assuming a MW = 280,000; see below). These values are much lower than expected for homogeneous enzyme samples but several characteristics of the reaction catalyzed could nevertheless be determined with preparations in hand.

The appearance of 4-Megln synthetase activity in extracts of seeds as a function of time of germination was determined from the amount of activity precipitated by ammonium sulfate. The activity appears to be absent in dormant seeds, rises to its maximum level after 5 to 7 days, and declines somewhat by 9 days at which time cotyledons begin to shrivel with advanced seedling development and onset of photosynthesis. Preparations obtained from whole seedlings showed much less activity of poorer stability than that from cotyledons alone. Accordingly, cotyledons detached from 5 to 7-day germinated seeds were used routinely for enzyme isolation. Although the level of activity

TABLE I: Requirements for Synthesis of 4-Methyleneglutamine, as Catalyzed by Partially Purified 4-Megln Synthetase

Reaction mixture	4-Megln formed (nmoles)	Glutamate released from glutamine (nmoles)
Complete[1]	220	---
$-NH_4^+$	0	---
-ATP	0	---
-4-Meglu	0	---
-Enzyme	0	---
$-NH_4^+$; + Gln	46	183
$-NH_4^+$,-4-Meglu; + Gln	0	117
$-NH_4^+$,-ATP; + Gln	0	89
-Enzyme, $-NH_4^+$; Gln	0	8.5

[1]Complete reaction mixtures contained in 0.2 ml: Tricine, pH 7.5, 20 μmol; $MgSO_4$, 4 μmol; ATP, 1.6 μmol; phosphocreatine, 2 μmol; creatine phosphokinase, 3 units; NH_4Cl, 2 μmol; L-4-Meglu, 4 μmol; and enzyme sample, 0.1 ml (8.5 munits). Where indicated, 2 μmol gln were added. Reactions were incubated 60 min at 37°C, heated in boiling water 3 min, 50 nmol α-aminobutyric acid added as an internal standard, and centrifuged. Aliquots of the supernatant fluid were analyzed on a Beckman 120C amino acid analyzer (2).

we currently detect in cotyledons is not adequate to account for the rapid rate of 4-Megln synthesis, the correspondence between the time of appearance of this new synthetase activity and the accumulation of 4-Megln in seedlings (2) strongly suggests a possible interrelationship.

Table I shows the requirements of this enzyme preparation for synthesis of 4-Megln. The apparent K_m for 4-Meglu was determined to be ~8 mM in three different assay systems, which value is 10-fold lower than that observed for the same substrate with peanut seedling glutamine synthetase (3). The enzyme appears to be relatively specific for NH_4^+ as amide donor; glutamine is about 20% as effective. Use of glutamine apparently involves direct transfer of amide rather than initial hydrolysis with release of NH_4^+ since the presence of 4-Meglu stimulates the release of glutamate in an amount approx. equal to that of 4-Megln synthesized.

Incubation of the enzyme with [8-^{14}C]ATP in the biosynthetic reaction mixture, followed by separation of ATP, ADP, and AMP on polyethyleneimine cellulose (6), showed that AMP formation was dependent on and approximately stoichiometric with 4-Megln synthesis (Table II). ADP formation, on the other hand, was not dependent on the presence of 4-Meglu and when phosphocreatine

TABLE II: Formation of [^{14}C]AMP and [^{14}C]ADP from [8-^{14}C]ATP in the 4-Meglu Synthetase Catalyzed Reaction

Experiment number	Assay mixture	[^{14}C]-Nucleotide Recovered		
		AMP (nmoles)	ADP (nmoles)	ATP (nmoles)
1.	Complete[1]	87.4	74.8	420
	+Phosphocreatine, creatine phosphokinase	129	9.4	479
	-4-Meglu	2.8	66.3	530
	$-NH_4^+$	4.1	80	518
	$-NH_4^+$; + Gln	15.8	91.4	512
	-Enzyme	0.7	19.2	587
2.	Complete	71.3	72.1	422
	-4-Meglu	1.8	80.5	499
	-4-Meglu; + Glu	29.6	157	370

[1]Complete reaction mixtures, incubation time, and mode of terminating reactions were the same as for Table I, except that: a) all amounts were halved; b) 0.2 µCi [8-^{14}C]ATP was included; c) the ATP-regenerating system was normally omitted; d) α-aminobutyric acid was not added. Where indicated, glu replaced the same concentration of 4-Meglu.

and creatine phosphokinase were included in the reaction mixture, both AMP production and 4-Megln synthesis were slightly stimulated. If 4-Meglu was replaced by glutamic acid, ADP production mainly was stimulated which would normally suggest the presence of glutamine synthetase activity. Enzyme preparations used, however, were free of conventional glutamine synthetase activity, as determined by the transferase assay system, leaving the question open for the present as to whether this new 4-Megln synthetase also utilizes glutamic acid to some extent via a different ATP-dependent route. GTP, UTP, and CTP, tested in place of ATP in the biosynthetic assay system did not catalyze any detectable formation of 4-Megln.

The effect of various amide donors and acceptors on AMP formation was also examined. With 4-Meglu as acceptor, hydroxylamine was 70% as effective as NH_4^+ whereas asparagine and methylamine were ineffective. When NH_4^+ was the donor, both glutamate and aspartate stimulated AMP formation but at 15% and 10%, respectively, the rate with 4-Meglu. With aspartate as acceptor and glutamine as amide donor, 5% as much AMP was formed; a reaction of this nature is indistinguishable from typical plant asparagine synthetases (7). The presence of contaminating amounts of asparagine synthetase, however, would not seem to

explain this activity with aspartate since (a) the major peak of asparagine synthetase activity eluted from the column of DEAE-cellulose at a higher NaCl concentration (Fig. 1) and (b) greater AMP formation was stimulated by aspartate with NH_4^+ as the amide donor.

In addition to distinct chromatographic behavior on DEAE-cellulose and reaction properties, 4-Megln synthetase activity differs from glutamine synthetase in not being sensitive to the glutamine synthetase inhibitor, L-methionine sulfoximine, and in its inability to catalyze amide-hydroxylamine exchange with either glutamine or 4-Megln. On gel filtration columns, 4-Megln synthetase activity is eluted between glutamine and asparagine synthetases. On a calibrated column of Sepharose CL-4B, the activity is eluted at a position corresponding to a molecular weight of ∿280,000, compared with 400,000 for glutamine synthetase and ∿200,000 for asparagine synthetase activity.

The properties observed with partially purified enzyme preparations so far obtained are sufficiently distinct to permit the conclusion that a previously unrecognized enzyme characterized as 4-methyleneglutamate:ammonia ligase (AMP-forming) is present. This conclusion also seems warranted since the activity is relatively specific for 4-Megln synthesis and its level increases in germinating peanut cotyledons roughly in parallel with the rate of appearance of 4-Megln in this species. Complete characterization of the enzyme rests on obtaining it in a more highly purified state.

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