Evidence for a developmentally regulated prespore-specific glutamine synthetase in the cellular slime mould *Dictyostelium discoideum*

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The enzyme glutamine synthetase (GS) is described for the first time in *Dictyostelium discoideum*. The appearance of this enzyme is developmentally regulated. The level of activity is low in vegetative cells and increases more than threefold during differentiation. Furthermore this enzyme is shown to be differentially localized in prespore cells, the specific activity being approximately fourfold higher than in prestalk cells. The enzyme has a pH optimum of 7.8 and 8.2 in the γ -glutamyltransferase and γ -glutamylsynthetase assays, respectively, and a temperature optimum of 45 °C. Kinetic studies of GS revealed apparent K_m values of 5.9 mM, 0.009 mM and 8.6 mM for glutamine, ADP and NH_2OH , respectively, in the γ -glutamyltransferase assay, and of 2.2 mM, 0.12 mM and 0.64 mM for glutamate, ATP and NH_2OH , respectively, in the γ -glutamylsynthetase assay.

Keywords: *Dictyostelium discoideum*, glutamine synthetase, ammonia assimilation

INTRODUCTION

The cellular slime mould *Dictyostelium discoideum* is a free-living soil micro-organism (Sussman & Bradley, 1954). Initially cells grow as single-celled amoebae which phagocytose and feed on bacteria. Upon depletion of the bacterial food source or amino acid starvation, amoebae undergo an aggregation process in response to pulses of cAMP and enter a developmental sequence that results in the formation of a fruiting body. The fully differentiated fruiting body consists of two cell types: spore cells with the capacity to germinate into new vegetative amoebae, and non-viable stalk cells (Loomis, 1988).

Development in *D. discoideum* is a highly coordinated process, in which signalling between the cells seems to control both their differentiation into stalk and spore cells and the overall morphogenesis of the aggregate (Berks *et al.*, 1991). A number of diffusible molecules of low molecular mass (morphogens) have been shown to play key roles in regulating development in *D. discoideum*. These include cAMP, differentiation-inducing factor (DIF), adenosine and ammonia (for a review see Williams, 1988). Ammonia has been shown to have a number of effects. It delays aggregation in aggregation-competent cells by lowering the overall level of intracellular cAMP

(Schindler & Sussman, 1977, 1979; Thadani *et al.*, 1977; Williams *et al.*, 1984) thus inhibiting terminal differentiation. It has also been shown to inhibit DIF accumulation (Neave *et al.*, 1983) and to act antagonistically to DIF by inducing genes specific to the prespore pathway and repressing expression of genes specific to the stalk pathway (Sternfeld & David, 1979; Gross *et al.*, 1983; Oyama *et al.*, 1988). Furthermore ammonia has been shown to switch cells from the prestalk to the prespore pathway (Bradbury & Gross, 1989).

On this basis it might be expected that the concentration of ammonia would be higher in prespore cells. However this is not the case; the level of ammonia is higher in prestalk cells (Feit reported in Cotter *et al.*, 1992; Rutherford *et al.*, 1982). A possible explanation for this apparent paradox is found in a model suggested by Insall *et al.* (1992). In this model once a group of cells begin to respond to a morphogen, then two events occur: (a) an increase in the sensitivity of the target cells to the morphogen and (b) removal of the morphogen. The combined effect of these two events is to prevent the morphogen rising to levels where it affects cells other than the initial group of target cells while allowing these cells to progress along the differentiation pathway.

In the case of ammonia, Cotter *et al.* (1992) extended this concept by proposing a source and sink model that predicts once differentiation has commenced ammonia production should occur in prestalk cells (i.e. the non-

Abbreviations: BAL, 2,3-dimercapto-1-propanol; DIF, differentiation-inducing factor; FITC, fluorescein isothiocyanate; GS, glutamine synthetase; MSX, methionine sulfoximine.

responsive group) and removal in the prespore cells (target group). These predictions are supported indirectly by a number of observations. Firstly there is extensive degradation of cellular macromolecules and therefore presumably release of ammonia in prestalk/prespore cells during differentiation, and secondly prespore cells accumulate large amounts of amino acids, especially glutamine and glutamate (Kelly *et al.*, 1979; Klein *et al.*, 1990).

It has been shown that both the NADH-dependent and the NADPH-dependent glutamate dehydrogenases exist in *D. discoideum* (Langridge *et al.*, 1977; Pamula & Wheldrake, 1990, 1991). During both vegetative growth and prestalk-stalk development it is likely that the NADH-dependent glutamate dehydrogenase functions primarily to degrade glutamate from protein catabolism to 2-oxoglutarate and NH_3 . In prespore-spore development either the NADH- or NADPH-dependent glutamate dehydrogenase may function to remove NH_3 via assimilation with 2-oxoglutarate to produce glutamate (Cotter *et al.*, 1992). Furthermore, given the very high levels of free glutamine found in spores (Klein *et al.*, 1990), ammonia may additionally be removed via the enzyme glutamine synthetase (L-glutamate ammonia ligase, ADP forming; EC 6.3.1.2) (GS) which catalyses the biosynthesis of glutamine from ammonia:

It is clear that the GS would be expected to play an important role in the removal of ammonia and the generation of an ammonia gradient. In this paper we demonstrate the presence of this enzyme in *D. discoideum*, describe some of its properties and examine its developmental regulation and cell type distribution.

METHODS

Reagents. L-Glutamine, L-glutamate, γ -glutamyl monohydroxamate, L-methionine sulfoximine (MSX), Pronase and 2,3-dimercapto-1-propanol (BAL) were from Sigma. ATP, ADP, and anti-mouse Ig-fluorescein were from Boehringer-Mannheim. Percoll was from Pharmacia and hydroxylamine from BDH. Mouse anti-*D. discoideum* spore serum was a kind gift from Professor K. L. Williams (Macquarie University, Sydney, Australia). Media components were purchased from Oxoid.

Culture conditions. *D. discoideum* (strain V12) were grown in liquid co-culture in KK2 buffer at 22 °C, using *Enterobacter aerogenes* as a food source, to $3\text{--}5 \times 10^6$ cells ml^{-1} (Pamula & Wheldrake, 1988). Cells washed three times in ice-cold KK2 buffer to free them of bacteria were used for the preparation of growth-phase (vegetative) cells. *D. discoideum* strain AX3 was grown in HL5 medium as described by Sussman (1987). To obtain cells at various stages of morphogenesis, washed vegetative amoebae were resuspended in KK2 to a density of 5.5×10^8 cells ml^{-1} and allowed to develop for selected times on cellulose nitrate membrane filters (4.5 cm diameter, Whatman), resting on supporting filter pads saturated with KK2 at 22 °C. Both vegetative and differentiating-stage cells were harvested by centrifugation at 200 *g* (5 min, 4 °C), washed three times in KK2, snap-frozen in liquid nitrogen and stored at -20 °C until required.

Isolation of prestalk and prespore cells. Separation of prestalk and prespore cells was based on a modified method of Tsang & Bradbury (1981). Briefly, vegetative cells prepared as described above were collected and allowed to form slugs at 22 °C on 1.5% (w/v) KK2 agar. Slugs were harvested in cold KK2 and centrifuged at 200 *g* (5 min, 4 °C) and the resulting cell pellet was resuspended in 10 vols dissociation buffer (0.1% Pronase, 25 mM BAL in 50 mM Tris/HCl, pH 7.0). Slugs were then dissociated after 30 min at room temperature by passing them four times through a 25 gauge needle. Dissociated slug cells were then filtered through stainless steel mesh (25 μm pore size) layered onto a preformed (30000 *g*, 10 min) gradient of 70% (v/v) Percoll (containing 20 mM potassium phosphate, 2 mM EDTA, 0.65% NaCl, pH 6.9) and centrifuged at 400 *g* (10 min, 4 °C). Resulting prestalk (light fraction) and prespore (heavy fraction) cells were carefully removed from the gradient and resuspended in 5 vols KK2. Prestalk cells and prespore cells were collected by centrifugation at 2500 *g* (10 min, 4 °C) and 400 *g* (5 min, 4 °C) respectively. To confirm that the exposure of slugs to BAL/Pronase did not effect GS activity, slugs were incubated in the presence and absence of BAL/Pronase at room temperature for 30 min and GS activity subsequently determined. The effectiveness of the separation of the two cell types was assessed by incubating cells with mouse anti-*D. discoideum* spore serum, followed by FITC-conjugated sheep anti-mouse IgG by the method of Durston *et al.* (1984). The ratio of fluorescent to non-fluorescent cells was determined by scoring on a Zeiss fluorescence microscope.

Preparation of cell extracts. All the following operations were performed at or below 4 °C. Cell pellets were resuspended in 10 vols 50 mM HEPES (pH 7.8) and disrupted by sonication as previously described (Dunbar & Wheldrake, 1994). The homogenate was centrifuged at 100000 *g* (20 min) to remove cellular debris. The resulting supernatant was desalted by passage through Sephadex G-25 M (PD-10, Pharmacia), filtered through a 0.22 μm filter (Millex-GV, Millipore) and retained for all experiments. Protein concentration of the extract was determined by the method of Bradford (1976) using BSA (Fraction V) as the standard.

Enzyme assay. GS activity was measured spectrophotometrically using the γ -glutamyltransferase and γ -glutamyl-synthetase assays described by Meister (1985). γ -Glutamyltransferase activity was followed by measuring the formation of γ -glutamyl monohydroxamate in the reaction mixture which contained in a final volume of 1 ml: 50 mM HEPES (pH 7.8), 40 mM glutamine, 30 mM sodium arsenate, 1 mM MnCl_2 or 20 mM MgSO_4 , 30 mM HCl/hydroxylamine neutralized just before use, 0.1 mM ADP and an appropriate amount of enzyme. The reaction was initiated by the addition of either ADP or enzyme and incubated at 30 °C for 30 min. γ -Glutamyltransferase activity in the absence of glutamine was used as the control. γ -Glutamylsynthetase activity was followed by measuring the formation of γ -glutamyl monohydroxamate in the reaction mixture which contained in a final volume of 1 ml: 50 mM HEPES (pH 8.2), 100 mM glutamate, 20 mM MgSO_4 , 30 mM HCl/hydroxylamine neutralized just before use, 10 mM ATP and an appropriate amount of enzyme. The reaction was initiated by the addition of either ATP or enzyme and incubated at 30 °C for 30 min. γ -Glutamyltransferase activity in the absence of glutamate was used as the control. γ -Glutamyl monohydroxamate was colorimetrically determined by addition of 1 ml ferric chloride reagent (370 mM FeCl_3 , 200 mM TCA and 670 mM HCl). Samples were centrifuged at 12000 *g* (3 min) to remove precipitated material. The A_{540} of the supernatant was measured after 20 min and the amount of γ -glutamyl monohydroxamate calculated by reference to a stan-

dard curve constructed with commercial γ -glutamyl monohydroxamate. The specific activity of GS is expressed in nmol γ -glutamylmonohydroxamate min^{-1} (mg protein) $^{-1}$.

RESULTS

GS activity in *D. discoideum* crude extracts

GS activity could be detected in crude extracts from *D. discoideum* using either the γ -glutamyltransferase or the γ -glutamylsynthetase assay. GS activity catalysing the Mg^{2+} -dependent γ -glutamyltransferase reaction showed an absolute requirement for Mg^{2+} and ADP. Less than 7% γ -glutamylmonohydroxamate was produced in the absence of either NH_2OH , arsenate or glutamine. Similarly in the absence of either Mg^{2+} or ATP no GS activity was detected in the γ -glutamylsynthetase reaction and less than 4% γ -glutamylmonohydroxamate was produced in the absence of either NH_2OH or glutamate. γ -Glutamyltransferase activity was approximately fivefold higher when Mg^{2+} was replaced by Mn^{2+} as the divalent cation. All of the components of the Mn^{2+} -dependent γ -glutamyltransferase reaction were required for the production of γ -glutamylhydroxamate except that activity in the absence of ADP was approximately 70% of that of the complete assay mixture. Thus unlike the Mg^{2+} -dependent activity the reaction catalysed by Mn^{2+} did not have an absolute requirement for ADP but was stimulated by approximately 30% on addition of this nucleotide. In the γ -glutamylsynthetase reaction Mn^{2+} did not support GS activity.

To confirm the GS activity was not of bacterial origin the enzyme assays were performed using *D. discoideum* AX3 cells grown in axenic culture and similar results were obtained (data not shown).

Effect of MSX

Apart from GS, γ -glutamylhydroxamate formation can also be catalysed by other enzymes such as glutaminases, amidotransferases (Meister, 1980) and γ -glutamyltranspeptidases (Orlowski & Meister, 1970). To confirm that the formation of γ -glutamylhydroxamate is a function of GS and not some other enzyme activity Mn^{2+} -dependent γ -glutamyltransferase and γ -glutamylsynthetase activities were measured in the presence of the specific GS inhibitor MSX (Genetet *et al.*, 1984). In the presence of MSX both activities decreased with increasing MSX up to 5.0 mM (Fig. 1). 50% inhibition was apparent at approximately 0.1 mM. A similar result was obtained for the Mg^{2+} -dependent γ -glutamyltransferase reaction (data not shown).

Kinetic properties of GS

The apparent pH optimum measured by both the Mn^{2+} (Mg^{2+})-dependent γ -glutamyltransferase assay and the γ -glutamylsynthetase assay was 7.8 and 8.2 respectively. The temperature optimum for both assays was 45 °C. At temperatures above 45 °C, thermal inactivation caused a rapid decrease in GS activity (data not shown).

Fig. 1. Effect of MSX on GS activity. Increasing concentrations of MSX were included in the Mn^{2+} -dependent γ -glutamyltransferase (●) and γ -glutamylsynthetase (○) reaction mix containing enzyme and incubated at 30 °C for 5 min before the reaction was initiated with ADP. Each point on the graph represents the mean of triplicate determinations. For Mn^{2+} -dependent γ -glutamyltransferase activity, 100% = 79.5 nmol min^{-1} (mg protein) $^{-1}$; for γ -glutamylsynthetase activity, 100% = 11.7 nmol min^{-1} (mg protein) $^{-1}$. This experiment was repeated twice with similar results.

All subsequent assays were done under optimum conditions except that the temperature was 30 °C, which is close to the growth temperature for *D. discoideum*. For the determination of Michaelis–Menten constants for the γ -glutamyltransferase and γ -glutamylsynthetase activities, one substrate concentration was varied while the others were kept near saturation. In the Mg^{2+} -dependent γ -glutamyltransferase assay the K_m values for glutamine, ADP, and NH_2OH were 5.9 mM, 0.009 mM, and 8.6 mM respectively. Identical K_m values were obtained for the Mn^{2+} -dependent γ -glutamyltransferase assay. For the γ -glutamylsynthetase assay the K_m values for glutamate, ATP and NH_2OH were 2.2 mM, 0.12 mM and 0.64 mM, respectively.

Developmental time course

In order to determine the role GS may play during development in the generation of an ammonia gradient, GS activity was determined at 4 h intervals following the onset of differentiation (Fig. 2). GS activity was found to rise very sharply at the late pseudoplasmodium stage (16 h) and peak at the Mexican hat stage (20 h) before declining with the formation of the fruiting body. Sonication may not release all of the enzyme at various stages of differentiation, in particular the last stages of culmination and fruiting body maturation. Therefore it was important to confirm that the changes in GS activity were not a result of differential release of the enzyme following sonication. To do this, cell extracts were prepared from all stages of development either by glass-bead grinding (Sigma type 1, 75–105 μm) or by Dounce homogenization. All three extraction techniques gave very similar results.

Fig. 2. Developmental time course of GS activity. Crude extracts were prepared from cells harvested at 0, 4, 8, 12, 16, 20, and 24 h after the onset of differentiation as described in Methods. At each time point GS activity was determined using both the Mn^{2+} -dependent γ -glutamyltransferase (●) and γ -glutamylsynthetase (○) assays. Each point on the graph represents the mean of triplicate determinations $\pm$ SEM. This experiment was repeated three times with similar results.

Table 1. Cell type distribution of GS activity

Prestalk and prespore cells were separated as described in Methods and GS activity was determined using both the Mn^{2+} -dependent γ -glutamyltransferase and γ -glutamylsynthetase assays. Each experiment contained three separate preparations of each cell type and for each cell type assays were performed in triplicate. All values presented are the means of triplicate determinations of each cell type $\pm$ SEM

Expt no.	Cell type	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	
		γ -Glutamyl- transferase	γ -Glutamyl- synthetase
1	Prestalk	22.13 $\pm$ 0.22	2.16 $\pm$ 0.34
	Prespore	91.62 $\pm$ 1.13	8.23 $\pm$ 0.71
2	Prestalk	22.26 $\pm$ 1.41	2.10 $\pm$ 0.49
	Prespore	93.82 $\pm$ 4.21	7.98 $\pm$ 0.85
3	Prestalk	23.12 $\pm$ 0.77	2.71 $\pm$ 0.14
	Prespore	89.53 $\pm$ 0.22	8.39 $\pm$ 0.22

Cell type distribution

The source and sink model (Cotter *et al.*, 1992) predicts that ammonia is removed from prespore cells via the biosynthesis of glutamine and subsequently there should be differential localization of GS in prespore and prestalk cells. In order to test this hypothesis, prestalk and prespore cells were separated and GS activity was determined in the two cell types (Table 1). In three separate experiments GS activity was found to be approximately fourfold higher in prespore cells than in prestalk cells. Immunofluorescent

staining of cells with an antibody highly specific to prespore cells conjugated to FITC-labelled sheep anti-mouse IgG was used to determine the purity of separation of the two cell types. The range of contamination of the prestalk fraction by prespore cells was routinely 5–10% (data not shown). The GS activity of the prestalk cells shown in Table 1 has been corrected for the contamination. Recently Bonner (1993) has shown that proteolytic enzymes such as papain may result in ammonia production in slug tissue over short time periods. The relevant control experiment (see Methods) indicates that the exposure of slugs to BAL/Pronase does not affect GS activity (data not shown) and that the observed values of GS activity are a true measure of native GS levels unaffected by experimental manipulation.

DISCUSSION

During the morphogenesis of *D. discoideum*, the level of ammonia rises as protein-rich unicellular amoebae are induced to turn over their proteins during the formation of multicellular fruiting bodies. In many organisms, particularly mammals, the level of free ammonia is controlled by GS (Mathews & Van Holde, 1990). Given the high levels of glutamine present in dormant spores of the *Dictyostelium* (Klein *et al.*, 1990) one possible role for GS may be to control the levels of free ammonia during differentiation.

This paper reports the presence of GS in *D. discoideum*. The enzyme could use either Mg^{2+} or Mn^{2+} as the divalent cation in the γ -glutamyltransferase assay, although Mn^{2+} gave an approximately fivefold higher activity than Mg^{2+} . The Mn^{2+} -dependent γ -glutamyltransferase activity was approximately ninefold higher than the Mg^{2+} -dependent γ -glutamylsynthetase activity. Similar findings have been reported for GS from a variety of organisms, including the basidiomycete fungi *Pleurotus ostreatus* (Mikes *et al.*, 1994) and *Stropharia semiglobata* (Schwartz *et al.*, 1991).

A significant amount of γ -glutamylmonohydroxamate was produced in the Mn^{2+} -dependent γ -glutamyltransferase reaction in the absence of ADP. A similar effect has previously been observed in *Anabaena cylindrica* (Sawhney & Nicholas, 1978). The requirement for both Mn^{2+} and arsenate and inhibition by MSX indicated that this reaction was catalysed by GS as opposed to other possible contaminants such as glutaminases, amidotransferases (Meister, 1980) and γ -glutamyltranspeptidases (Orlowski & Meister, 1970). In the Mg^{2+} -dependent γ -glutamyltransferase reaction no γ -glutamylmonohydroxamate was produced in the absence of ADP. Thus the ADP-independent activity does not appear to be due to ADP bound to the enzyme. It is possible that Mg^{2+} and Mn^{2+} stabilize different conformational states of the enzyme and the one induced by Mn^{2+} is partially active in the absence of added ADP.

The relatively high optimum reaction temperature for both the γ -glutamyltransferase and γ -glutamylsynthetase activities is in agreement with the values from other sources (Canovas *et al.*, 1984; Ertan, 1992; Shatters *et al.*,

1993). The reported K_m values are apparent values since the enzyme has not yet been purified. The K_m values do however confirm that the concentrations of reactants used in both assays were in excess and not rate-limiting. The K_m values for ATP and glutamate in the γ -glutamyl-synthetase assay are similar to the concentrations of ATP (0.7 mM) (Wright & Kelly, 1981) and glutamate (2.3 mM) (Kelly *et al.*, 1979) in differentiating *D. discoideum* cells. It is likely then that GS plays a physiological role in the conversion of ammonia to glutamine.

GS from *D. discoideum* is developmentally regulated. Late differentiation-stage cells (Mexican hat) have an approximate threefold increase in activity compared to vegetative stage cells. The rise in GS activity and the concomitant rise in glutamine (Klein *et al.*, 1990) may serve a number of functions: firstly to allow the generation of an ammonia gradient; secondly, to avoid nitrogen loss (in the form of ammonia) since this is a scarce resource in soil environments; and thirdly to accumulate reserves of readily available nitrogen required for spore germination (Klein *et al.*, 1990).

GS activity has been shown to be differentially localized in prespore cells, suggesting that GS is a late prespore induced enzyme that is also present in vegetative cells. A number of questions arise from this. Firstly, is the vegetative enzyme the same as the differentiating enzyme? In plants, for example, there are cytosolic (GS1) and plastidic (GS2) isoenzymes of GS (McNally & Hirel, 1983), and in bacteria three types of GS, termed GSI, GSII, and GSIII have been described (Woods & Reid, 1993). It is therefore possible that separate vegetative and differentiating GS isoenzymes may exist. Secondly, what level of control operates on the enzyme throughout differentiation? These questions are currently under investigation.

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