INHIBITION OF THE ADENYLYLATION OF GLUTAMINE SYNTHETASE BY METHIONINE SULFONE DURING NITROGENASE DEREPRESSION

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SUMMARY

Adenylylation of glutamine synthetase was suppressed during derepression of nitrogenase synthesis in the presence of methionine sulfone and an excess of NH_4^+. Deadenylylation of glutamine synthetase was also promoted during nitrogenase derepression under the same conditions. These results are consistent with the hypothesis that the unadenylylated form of glutamine synthetase is required for derepression of nitrogenase.

INTRODUCTION

Regulation of nitrogenase synthesis in free-living nitrogen-fixing bacteria has been the focus of several recent reports. Gordon and Brill (1) demonstrated that L-methionine sulfone (MSF) and L-methionine-DL-sulfoximine (MSX), which inhibit both glutamine synthetase and glutamate synthase, derepress nitrogenase synthesis by Klebsiella pneumoniae and Azotobacter vinelandii when grown in media containing an excess of NH_4^+. Both Tubb (2) and Streicher et al. (3) have presented evidence indicating that catalytically active glutamine synthetase is necessary for nitrogenase synthesis. They also have reported that strains of Klebsiella aerogenes and K. pneumoniae that exhibit the $GlnC^-$ mutant phenotype (constitutive synthesis of glutamine synthetase) and carry the nif operon(s) were partially derepressed for nitrogenase synthesis when cultured in the presence of NH_4^+. These results suggest that glutamine synthetase may act as a positive control element for nitrogenase synthesis in a manner similar to that proposed for histidase synthesis in K. aerogenes (4).

Recently, Tyler et al. (5) have shown that in vitro transcription of the correct strand of DNA coding for the histidine utilization operon from Salmonella typhimurium is activated by unadenylylated glutamine synthetase and not by the

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adenylylated form. Activation of transcription of the nif operon has been inferred to take place in an analogous way (2). From this we thought that the structural analogs of glutamate, MSF and MSX might suppress adenylylation of glutamine synthetase thereby inducing derepression of nitrogenase in the presence of excess NH_4^+.

Our results indicate that adenylylation of glutamine synthetase is indeed suppressed in and deadenylylation accelerated by cells supplied with MSF and an excess of NH_4^+. A preliminary account of this investigation has been presented (6).

MATERIALS AND METHODS

Cultural Conditions

Klebsiella pneumoniae, strain M5al utilized in the experiments described in Tables I and II, were cultured by the following procedure. Two hundred ml of a sucrose minimal medium (7) supplemented with 400 µg N per ml (as ammonium acetate) were inoculated from a streaked nutrient agar plate and incubated with vigorous shaking at 30°C for 24 hr. One hundred ml of this culture was added to 900 ml of fresh medium. After 19 hr of vigorous shaking at 30°C the culture contained 5×10^9 viable cells per ml. At this time each culture was centrifuged and the cells resuspended in 1 l. of fresh N-free minimal medium (7) and incubated with vigorous shaking for 2 hr.

Acetylene Reduction Assay

The in vivo nitrogenase activity of cultures was measured by the acetylene reduction technique. In these experiments a 1.8 ml-sample of each culture was transferred with a syringe to a stoppered 21 ml serum vial containing 0.2 ml of a solution of degassed phosphate buffer and 2% sucrose.

The gas phase consisted of 0.81 atm of argon. The assay was initiated by injection of 2.0 cm^3 of acetylene into each vial. After incubation of the vials for 30 min at 30°C, a 0.5 cm^3 sample was withdrawn from each vial and assayed for acetylene reduction by the gas chromatographic technique as described by Burris (8). Whole cell protein was determined as described by Prival et al. (4).

Preparation of Crude Extracts

Crude extracts were prepared by suspending packed cells in an equal volume of 10 mM imidazole-HCl buffer (pH 7.0) containing 1 mM $MnCl_2$ (imidazole-Mn^{2+} buffer) and then by exposure at 0-4°C to three 30-second periods of sonication. Cell debris was removed by centrifugation at 40,000 xg for 30 min. The protein content of the extracts was determined by the Lowry method (9).

Assay for Glutamine Synthetase Activity

Glutamine synthetase activity was measured by the α-glutamyl transferase assay in the presence of 0.3 mM Mn^{2+} using the procedure outlined by Shapiro and Stadtman (10). Specific activity is defined as µmoles of α-glutamyl hydroxamate produced per mg of protein per min at 30°C.

TABLE I. THE EFFECT OF MSF AND NITROGEN SOURCE ON ADENYLYLATION OF GLUTAMINE SYNTHETASE

An aliquot (175 ml) of a suspension of K. pneumoniae cultured as described under Materials and Methods was transferred to each of three sterile 300 ml suction flasks. To one was added sufficient ammonium acetate to obtain a concentration of 400 µg N/ml. To the second flask was added L-methionine sulfone (from Sigma Chemical Co.) at a concentration of 2 mg per ml, and ammonium acetate (400 µg N/mg). The third flask to which no addition was made served as an N_2 control. The relative adenylylation and average state of adenylylation of glutamine synthetase present in extracts of the added cells were 1.0 and $\bar{E}0$ respectively. The data presented in the table below were obtained after a 5 hr incubation period during which each 175 ml culture was sparged vigorously with sterile N_2 of high purity.

Experimental Conditions

Nitrogen source	L-Methionine sulfone added (mg/ml)	Glutamine synthetase (Sp. act.*)	Relative adenylylation**	Average state of adenylylation $\bar{E}n$**
		Experiment 1		
N_2	none	0.18	1.0	ND***
NH_4^+	none	0.34	3.6	ND
NH_4^+	2.0	0.25	1.1	ND
		Experiment 2		
N_2	none	0.17	1.0	0.0
NH_4^+	none	0.30	3.4	8.3
NH_4^+	2.0	0.60	1.7	3.7

Note: Footnotes on following page.

Determination of Relative Adenylylation and the Average State of Adenylylation of Glutamine Synthetase

Relative adenylylation of glutamine synthetase in crude extracts was determined by a modification of the snake venom phosphodiestrase (SVD) method described by Tronick et al. (11). To a given volume of each crude extract was added an equal volume of 1% streptomycin sulfate in imidazole-Mn^{2+}. After 30 min at 0-4°C the streptomycin precipitate was removed by centrifugation at 10,000 xg for 10 min. Each supernatant fraction was dialyzed for 15 hr at 0-4°C against 100 volumes of imidazole-Mn^{2+} buffer (pH 7.0) and then centrifuged at 10,000 xg for 10 min. Each dialyzed extract was diluted with imidazole-Mn^{2+} buffer so that an addition of an 0.05 ml aliquot to the glutamine synthetase transferase assay (containing 60 mM Mg^{2+} and 0.3 mM Mn^{2+}) resulted in an absorbance of about 0.15 at 540 nm. Each relative adenylylation determination was conducted by adding a 0.05 ml aliquot of the diluted extract to each of 4 tubes. Two of these received 0.1 ml of Tris-acetate buffer (pH 8.8) containing 15 mM magnesium acetate and the other two tubes received 0.1 ml of the Tris acetate-magnesium acetate buffer containing 30 µg of SVD (snake venom phosphodiestrase from Nutritional Biochemical Corp.). SVD-treated and non-treated samples were removed after incubation periods of 60 and 120 min at 37°C, and glutamine synthetase transferase activity was determined in the presence of 60 mM Mg^{2+} and 0.3 mM Mn^{2+}. The ratio of the absorbancies at 540 nm resulting from the transferase assay of an SVD-treated sample versus that of a non-treated sample is reported as the relative adenylylation value. A relative adenylylation of 1.0 indicates the absence of adenylylated enzyme, and a value greater than 1.0 indicates the presence of adenylylated enzyme. The average state of adenylylation ($E\bar{n}$), of glutamine synthetase in crude extracts, prepared as described for relative adenylylation estimates was determined by the method outlined by Stadtman et al. (12) with the exception that the transferase assays were conducted at pH 7.60 (the apparent isoactivity point for the Klebsiella enzyme, unpublished results) instead of pH 7.15 (the isoactivity point for the E. coli enzyme). As stated by Stadtman et al. (12) the value of $\bar{n}$ may vary from 0 to 12 and it represents the number of equivalents of adenyl groups per mole of enzyme from Escherichia coli. The quantitative relationship between $\bar{n}$ values for glutamine synthetase from K. pneumoniae and equivalents of adenyl groups per mole of enzyme has not been firmly established, however, there is no reason to believe that the relationship differs greatly from that established with the glutamine synthetase from E. coli.

RESULTS AND DISCUSSION

Preliminary experiments indicated that the glutamine synthetase transferase activities of extracts of cells treated with MSX were too low for the determina-

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* Mean of duplicate determinations of specific activity (µmole of α-glutamyl hydroxamate produced/mg protein per min at 30°C). Standard errors of the mean ranged from 1 to 11% of values.

** See Materials and Methods section for definitions.

*** Not determined.

TABLE II. THE EFFECT OF MSF ON DEADENYLYLATION OF PARTIALLY ADENYLYLATED GLUTAMINE SYNTHETASE

Cultural conditions were as outlined for the experiments described in Table I except that the cultures were incubated anaerobically with NH_4^+ for 2 hr prior to addition of the MSF in order to promote adenylylation of glutamine synthetase. Initial relative adenylylation values of 2.6 and 1.7 were found for Expts. 1 and 2 respectively and the average state of adenylylation was $E_{3.3}$ for Expt. 2. The data presented in the table were obtained after 5 hr of anaerobic incubation.

Experimental Conditions

Nitrogen source	L-Methionine sulfone added (mg/ml)	Relative adenylylation	Average state of adenylylation $\bar{En}$*
		Experiment 1	
NH_4^+	none	4.5	ND**
NH_4^+	2.0	1.2	ND
		Experiment 2	
NH_4^+	none	3.1	7.9
NH_4^+	2.0	1.1	0.5

* See Materials and Methods section for definition.

** Not determined.

tion of reproducible relative adenylylation values. Extracts of cells treated with MSF, on the other hand, contained moderate levels of glutamine synthetase transferase activity from which reproducible relative adenylylation values could be determined. These results are not surprising because MSX is known to inhibit glutamine synthetase by binding irreversibly to the active site while MSF inhibits by reversibly binding to the active site (13). In this investigation, therefore, MSF was selected for detailed studies even though MSX has been reported to be more effective than MSF in alleviating NH_4^+-repression of nitrogenase synthesis (1).

The relative adenylylation of glutamine synthetase (Table I, Expts. 1 and 2) in crude extracts of cells supplied with N_2 as the sole nitrogen source was 1.0 whereas values of 3.6 (Expt. 1) and 3.4 (Expt. 2) were obtained for glutamine synthetase in extracts of cells supplied with NH_4^+. These values are also in good agreement with those obtained for the average state of adenylylation (Table I, Expt. 2). Thus, it is apparent that the unadenylylated form of glutamine synthetase is found in cells supplied with N_2 as the only nitrogen source while moderately adenylylated glutamine synthetase is present in cells supplied with NH_4^+. Values of 1.1 (Expt. 1) and 1.7 (Expt. 2) were determined for the relative adenylylation of the enzyme in extracts of cells incubated in the presence of MSF and supplied with NH_4^+ (Table I, Expts. 1 and 2). Again, the average state of adenylylation is in close agreement with the values obtained for relative adenylylation (Table I, Expt. 1). From these results it appears that MSF either directly or indirectly inhibits adenylylation of glutamine synthetase in the presence of excess NH_4^+.

The amount of nitrogenase activity observed in the presence of MSF and NH_4^+ was found to vary and ranged from a specific activity of 0.04-1.12 nmoles C_2H_2 reduced per mg cell protein per min. The level of nitrogenase derepression under these conditions was always greater, however, than that observed in the presence of NH_4^+, but without MSF.

Since the cells utilized to initiate the experiments shown in Table I contained unadenylylated glutamine synthetase it is possible that MSF could inhibit the adenylylating enzyme system directly without promoting deadenylylation of previously adenylylated enzyme. This possibility was examined by incubating 3 cultures anaerobically with NH_4^+ in order to promote adenylylation. After 2 hr of incubation, values of 2.6 (Expt. 1) and 1.7 (Expt. 2) were obtained for the relative adenylylation of glutamine synthetase in cells of one of the cultures. Of the remaining cultures, one served as a control while MSF was added to the other. After an additional 5 hr of incubation, the relative adenylylation values of glutamine synthetase in extracts of control cells were

4.5 (Expt. 1) and 3.1 (Expt. 2) as compared to 1.2 (Expt. 1) and 1.1 (Expt. 2) for the enzyme in extracts of MSF-treated cells (Table II, Expts. 1 and 2). In Expt. 2 of Table II, the average state of adenylylation correlated well with the relative adenylylation values. Thus, it appears that MSF may promote deadenylylation as well as inhibit adenylylation in the presence of high levels of NH_4^+. The possibility cannot be ruled out, however, that the unadenylylated enzyme may represent newly synthesized glutamine synthetase and not deadenylylation of preexisting adenylylated enzyme. Under these conditions the specific activity of nitrogenase in MSF-treated cells was 0.03 to 0.08 nmoles C_2H_2 reduced per mg cell protein per min and zero in the absence of MSF.

A possible explanation for the effect of MSF on adenylylation and deadenylylation in the presence of excess NH_4^+ is that MSF may decrease the intracellular level of glutamine by inhibiting glutamine synthetase. This lower level of glutamine would alter the α-ketoglutarate: glutamine ratio which in turn could suppress adenylylation and trigger deadenylylation of glutamine synthetase through an intricate cascade system (14).

The observations presented above are consistent with the proposal that the unadenylylated form of glutamine synthetase is that which acts as the postulated positive control element during derepression of nitrogenase synthesis. Furthermore, under all conditions where nitrogenase activity could be detected, relative adenylylation values were nearly 1.0 regardless of whether MSF was added to the culture or not (Bishop, P.E. and McParland, R.H., unpublished results).

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