INVOLVEMENT OF THE BIOSYNTHETIC PATHWAY OF PURINE NUCLEOTIDES IN THE REPRESSION OF BACTERIAL SPORULATION.

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Results reported here show that the repressive action of glutamine on sporulation described previously is brought about by those early steps of the purine nucleotide pathway in which glutamine is an amino group donor.

In Bacillus megaterium grown in glucose-ammonia minimal medium, ammonia is assimilated via the glutamate cycle. This cycle involves two enzymes, glutamine synthetase (GlnS) and glutamate synthase (GluS) (1). With the aid of mutants blocked either in GlnS or in GluS, it has been shown that glutamine and not glutamate brings about repression of sporulation (2). We now report that the repressive action of glutamine is mediated by the early steps of the purine nucleotide pathway.

MATERIALS AND METHODS

All bacterial strains were derived from B. megaterium MA 22 (Ura^-), an auxotrophic mutant that requires uracil for growth but not for sporulation. Isolation of Gln^-26 (Ura^-, $GlnS^-$) and of Glu^-11 (Ura^-, $GluS^-$) mutants has been previously described (1).

Mutations in the purine nucleotide pathway were localized by the following criteria : excretion of diazotizable compounds ; ability to utilize adenine, guanine, hypoxanthine, xanthine, 5-amino-4-imidazole carboxamide as growth factor ; thiamine requirement ; syntrophic growth. The Pur^- mutants were classified according to Magasanik (3) : class 1a, from 5-phosphoribosyl-1-pyrophosphate (PRPP) to 5-aminoimidazole ribonucleotide (AIR) ; class 1b, from AIR to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) ; class 1c,

from AICAR to inosinic acid (IMP) ; class 2 from IMP to xanthylic acid (XMP) ; class 3, from XMP to guanylic acid ; class 4, from IMP to adenylic acid.

The procedure for studying sporulation was similar to that described for MA 22 (4). Bacteria were grown in minimal medium (1) supplemented with growth factors as necessary. Before the end of growth cells were transferred to sporulation media at a final concentration of 25 x 10^6 per ml. Sporulation media consisted of the salts of the minimal medium to which were added none, one, or several of the following compounds : 11 mM glucose (G), 38 mM ammonium chloride (N) and growth factors at concentrations indicated in the text. In all media, at least one compound required for growth was omitted, so that bacteria would or would not sporulate depending on whether or not repression of sporulation was relieved.

Thermoresistant spores were counted 18 hrs after transfer by plating on nutrient agar aliquots previously heated at 70°C for 10 min. Percent sporulation was expressed as 100 time the ratio between the number of thermoresistant spores and the number of cells at the time of transfer.

RESULTS

1) Sporulation of MA 22 strain and of Gln 26 and Glu 11 mutants. Sporulation properties of the three strains have been reported in greater details elsewhere (2, 4). They are summarized in Table I which shows that sporulation of MA 22 is repressed in G+N+ medium and is derepressed in G+N- medium. Sporulation of Gln 26 escapes repression in G+N+ medium, whereas sporulation of Glu 11 is repressed even in G+N- medium. In agreement with our earlier report (2), addition of 200 µg glutamine per ml to either medium led to repression of sporulation of Gln 26, whereas the same concentration of glutamate was without effect.

2) Isolation and properties of Gln 26 S8 mutant. If glutamine is the precursor of a specific repressing compound, a mutant of Gln 26, selected for its ability to sporulate in G+N+ medium supplemented with glutamine, might be blocked in the synthesis of this

Table I. Percent sporulation of B. megaterium MA 22 and of Gln⁻ 26 and Glu⁻ 11 mutants.

	Sporulation media	
	G+N-	G+N+
MA 22	85	0.02
Gln⁻ 26	26	33
Glu⁻ 11	0.07	0.03

compound. UV mutagenized spores of Gln⁻ 26 were inoculated in complex medium (1), growing cells were transferred to G+N+ medium supplemented with 200 µg glutamine per ml. Spores formed in the G+N+ medium were submitted to the same cycle of growth and sporulation. After four cycles the rate of sporulation approached 100 %. The majority of the clones derived from the spore population were Ura⁺ revertants and approximately 1 % had one further auxotrophic mutation in addition to the Ura⁻ and GlnS⁻ markers of the mother strain. Of this latter category, we randomly selected eleven clones, ten of which required one amino-acid and one of which required a purine base. In G+N+ medium supplemented with glutamine, the mutants with an amino-acid requirement sporulated at a rate ranging from 0.5 to 4 %, whereas the mutant with the purine base requirement sporulated at a high rate. The latter designated Gln⁻ 26 S8 is a class Ia mutant, as defined above.

Results reported in Table II show that sporulation of Gln⁻ 26 S8 escapes repression in G+N+ medium whether or not supplemented with glutamine. Addition of uracil had no effect whether or not glutamine was present. Addition of adenine repressed sporulation only in the presence of glutamine. This suggests that the Pur⁻ mutation is responsible for the sporulation properties of Gln⁻ 26 S8.

3) Isolation and properties of Glu⁻ 11 S1 mutant. Repression of sporulation of Glu⁻ 11 mutant in both G+N+ and G+N- media (see Table I) may be attributed to the fact that Glu⁻ 11, as opposed to

Table 3

Hydrolysis of Tubercidin-, Toyocomycin-, and Sangivamycin 3',5'-Cyclic Phosphates by cAMP Phosphodiesterases

Compound	Relative Rate of Hydrolysis	
	Beef Heart	Rabbit Kidney
Adenosine 3',5'-cyclic phosphate	1.0	1.0
Tubercidin 3',5'-cyclic phosphate (4)	0.77	0.59
Toyocomycin 3',5'-cyclic phosphate (8)	0.53	0.39
Sangivamycin 3',5'-cyclic phosphate (12)	0.29	0.16

The cAMP phosphodiesterases were purified as described previously (4). The standard reaction mixture contained in 0.60 ml: 3.0 μmol cyclic nucleotide; 30 μmol tris·HCl, pH 7.5; 6 μmol $MgCl_2$; and phosphodiesterase protein (0.15 mg of rabbit kidney enzyme and 0.22 mg of beef heart enzyme). After an appropriate incubation period (10-60 min), the reaction was terminated by heating, treated with bacterial alkaline phosphatase, and the phosphate released was assayed colorimetrically as previously described (4). The actual rates of hydrolysis of cAMP were 45 and 32 nmol 5'-AMP formed per min for the kidney and heart enzymes, respectively.

rabbit lung and beef heart cAMP phosphodiesterases. The three cyclic nucleotides were all very good inhibitors. This is probably partly because they are serving as substrates for these enzymes. The nucleosides and nucleoside 5'-phosphates were in general quite poor inhibitors of the enzymes, while the heterocyclic bases were good inhibitors, but not as active as the cyclic nucleotide. That the nucleosides and nucleoside 5'-phosphates were poor inhibitors, while the cyclic nucleotides were good inhibitors, indicates that the ribose 3',5'-cyclic phosphate moiety is also a point of interaction between the cyclic nucleotide and the cAMP phosphodiesterases. Preliminary studies on the kinetics of inhibition of the rabbit lung and beef heart phosphodiesterases have shown that the 3',5'-cyclic nucleotides (4, 8, 12), 5'-nucleotides (3, 7, 11), and nucleosides (2, 6, 10) are all competitive inhibitors of both enzymes, while the heterocyclic bases (1, 5, 9) show a mixed type of inhibition. These

Table III. Percent sporulation of Pur$^-$ mutants

	Sporulation media			
	G+N-	G+N+		
		no addition	+ uracil (20 µg/ml)	+ adenine (20 µg/ml)
Pur 562	30	37	86	0.4
Pur 45	54	0.1	-	-
Pur 26	22	0.1	-	-
Pur 47	46	1.2	-	-
Pur 76	25	0.4	-	-

1a, should sporulate in the G+N+ medium. In addition we wondered what sporulation behavior would be exhibited by mutants belonging to other classes of the purine pathway. A series of Pur$^-$ mutants was isolated from UV mutagenized spores of MA 22. Representative mutants were obtained for each class except 1c. Five were studied further : Pur 562, class 1a ; Pur 45, class 1b ; Pur 26, class 2 ; Pur 47, class 3 ; and Pur 76, class 4.

Results reported in Table III show : a) all mutants sporulated in G+N- medium ; b) only Pur 562, which belongs to class 1a, sporulated in G+N+ medium ; c) sporulation of Pur 562 in G+N+ medium was repressed by addition of adenine, but not of uracil. The latter finding suggests that the sporulation properties of Pur 562 are not due to the Ura$^-$ mutation. This conclusion was confirmed by studying spontaneous revertants of Pur 562 for each of the two auxotrophic mutations. Pur 562 R3 revertant (Ura$^+$, Pur$^-$), like Pur 562, sporulated in G+N+ medium, but Pur 562 R1 revertant (Ura$^-$, Pur$^+$), like MA 22, did not sporulate in this medium.

DISCUSSION

The results reported above indicate that a mutation in a step of the purine pathway localized between PRPP and AIR brings

about derepression of sporulation in a medium containing both glucose and ammonia. However, a mutation in a step localized after AIR does not relieve repression of sporulation in such a medium. The only two reactions in which glutamine intervenes between PRPP and IMP in the purine pathway are localized before AIR. Therefore the previously reported repressive action of glutamine (2) is likely to be mediated by these reactions which either permit synthesis of a repressing compound or prevent synthesis of an inducing compound. No obvious explanation can account for the observation that sporulation repression of Pur^- class 1a mutants in G+N+ medium requires addition to the medium either of adenine alone if the mutant is $GlnS^+$ or of adenine and glutamine if the mutant is $GlnS^-$. It is not yet possible to go further in the analysis of the mechanism of sporulation repression. However additional study of the Pur 562 mutant should lead to a better understanding of this mechanism.

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