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The SpoIIAA Protein of *Bacillus subtilis* Has GTP-Binding Properties

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SpoIIAA is the first protein of the *spoIIA* operon. Here we show that SpoIIAA can bind and hydrolyze GTP. The protein also accepts ATP, but with lower affinity. GDP competes poorly for binding of GTP. The GTPase activity of SpoIIAA is within the range found for other GTP-binding proteins.

When bacilli face starvation, they begin a series of processes that culminate in the formation of a spore (26). Sporulation starts with an asymmetrical cell division, after which the daughter cells undergo differential gene expression through the specific activation of sigma factors in the two cells (9, 10, 19). It is known that the intracellular concentrations of GTP and GDP drop significantly whenever cells are induced to sporulate (18, 29). Chemicals such as decoyinine that cause the GTP concentration to fall induce sporulation even in rich medium (11). These results suggest that one or more regulatory GTP-binding proteins monitor the intracellular GTP concentration and adjust gene expression accordingly (26). A dozen GTP-binding proteins have been found in extracts of vegetative and early-sporulation cells of *Bacillus subtilis* (21), but it is not known which of them respond to the change in intracellular GTP concentration that leads to the initiation of sporulation.

Here we report that SpoIIAA, the first product of the tricistronic operon *spoIIA*, behaves like a GTP-binding protein in that it can bind and hydrolyze GTP. SpoIIAA has been studied for its role in the regulation of the prespore-specific sigma factor σ^F (1, 8, 24). In vitro experiments have shown SpoIIAA to be an anti-anti-σ^F protein which can interact with the anti-σ^F protein SpoIIAB (1, 8). The present results raise the possibility that SpoIIAA has an additional role as a GTP-binding protein.

SpoIIAA binds GTP. Direct photoaffinity labeling (UV cross-linking) (2), performed by a modification of the method of RayChaudhuri and Park (23), showed that SpoIIAA binds to GTP. Protein (20 μM), prepared as described in reference 8, was mixed with 1 μM [α-^{32}P]GTP (650 Ci/mmol; ICN) in buffer B (50 mM Tris-Cl [pH 7.5], 50 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 1 mg of bovine serum albumin [BSA] per ml) in a total volume of 50 μl. After preincubation on ice in the dark for 10 min, samples were exposed to UV (254 nm) from a handheld UV Mineralight at a distance of about 2 cm. The autoradiogram presented in Fig. 1a shows that the extent of protein labeling increased with time. The presence of 20 μM SpoIIAB (added together with 50 mM EDTA, which abolishes the phosphokinase activity of SpoIIAB) enhanced labeling of SpoIIAA (Fig. 1b). (The same experiment made it clear that SpoIIAB can also bind GTP. Recent experiments [data not shown] have shown that GTP can substitute for ATP in the phosphorylation of SpoIIAA catalyzed by SpoIIAB, and we presume that UV can cross-link GTP to SpoIIAB at the latter's nucleotide binding site.) Similar experiments showed that SpoIIAA can also cross-link with ATP, but with lower affinity than with GTP (data not shown).

Ultrafiltration with a Centricon 10 filter (Amicon) was used to measure the binding of GTP to SpoIIAA. SpoIIAA and [α-^{32}P]GTP (25 Ci/mmol; ICN) were mixed in buffer B (without BSA) in a total volume of 1 ml in a Centricon 10 at 35°C. After presaturation of the filter with radioactivity, the Centricon 10 was quickly centrifuged at 1,000 × *g* for 2 min at specified times. Counting of radioactivity in samples of the liquid that had passed through the filter and of the liquid that remained above the filter gave an estimate of the binding of the nucleotide to the protein. The maximum binding of GTP to SpoIIAA was reached by about 30 min and amounted to about 0.6 mol/mol. To determine the dissociation constant of the interaction between SpoIIAA and nucleotide, binding was measured at 35°C with a fixed protein concentration of 100 nM and different concentrations of nucleotide (Fig. 2). Assuming that all the protein in our preparation is active in binding, the results of this experiment suggest that one GTP molecule binds to a SpoIIAA dimer. Apart from the motif DXXG (the protein contains DSSG and DMSG), SpoIIAA lacks the conserved G-domain sequence elements reported for GTP-binding proteins (3, 7). One element that is normally conserved in both GTP- and ATP-binding proteins is the P loop, GXXXXGK (3, 7). Although the sequence GLGVILGR occurs in SpoIIAA, it deviates significantly from the P loop. Conceivably, dimerization of SpoIIAA could endow the molecule with the structure necessary for GTP binding.

From the equation $1/r = 1/n + K_d/n$[free GTP] (22), the dissociation constant of binding of SpoIIAA to GTP was found to be 0.25 μM. In a similar experiment, the dissociation constant of binding of SpoIIAA to ATP was about 2.5 μM, confirming that SpoIIAA has a higher affinity for GTP than for ATP. We note that most GTP-binding proteins studied to date show specificity for GTP and do not bind to ATP (3, 7, 25). Experiments with GDP and ADP showed that these nucleotides compete poorly with GTP and ATP for binding to SpoIIAA (results not shown).

SpoIIAA has GTPase and ATPase activities. SpoIIAA not only binds to but also hydrolyzes GTP and ATP. To measure the GTPase or ATPase activity, we used the procedure of de Boer et al. (6) with some modifications. SpoIIAA was incubated with nucleoside triphosphate (NTP) (containing 0.02 μM [γ-^{32}P]NTP; 4,500 Ci/mmol; ICN) in buffer B (without BSA) at 35°C. The reaction was stopped with 1.5% ammonium molybdate in 0.5 N sulfuric acid, and the radioactive P_i was extracted into isoamyl alcohol and counted. The GTPase activity of SpoIIAA was linear with time over at least 240 min.

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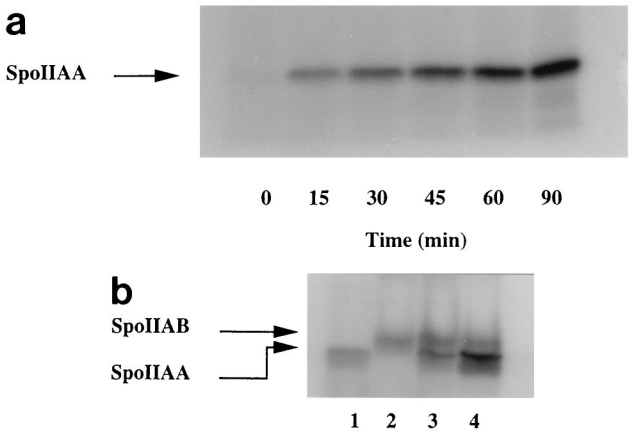

FIG. 1. (a) Time course of UV cross-linking of SpoIIAA with [α-^{32}P]GTP. The figure shows an autoradiogram of a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel. (b) Effect of SpoIIAB on UV cross-linking of SpoIIAA with [α-^{32}P]GTP. Incubation was for 20 min. Lane 1, SpoIIAA; lane 2, SpoIIAB; lane 3, SpoIIAA plus SpoIIAB; lane 4, SpoIIAA plus SpoIIAB plus 50 mM EDTA. The figure shows an autoradiogram of an SDS–15% polyacrylamide gel.

We measured the kinetic parameters of the NTPase for the two nucleotides GTP and ATP. The V_{max}s for GTPase and ATPase were 4.0 and 1.4 nmol min^{-1} mg^{-1}, respectively, and the K_ms for GTPase and ATPase were 3.3 and 3.8 μM, respectively. The GTPase activity of SpoIIAA falls within the range reported for some other GTP-binding proteins, being stronger than that of p21^{ras} (13, 27), EF-Tu (17), and Obg (30) but weaker than that of FtsZ (16) and the α subunit of heterotrimeric G proteins (12, 14). It should be noted that GTPase-activating proteins for Ras and EF-Tu have been discovered (17, 28). At present we do not know whether the GTPase activity of SpoIIAA is stimulated by another protein.

GTPase activity is not due to a contaminant in the SpoIIAA preparation. The GTPase activity in the preparation of SpoIIAA was destroyed by boiling and retained by a Centricon 10 filter (data not shown), suggesting that the GTPase activity must be due to a protein and not to, e.g., metal ion contamination. We did a series of experiments to exclude the possibility of contamination with an extraneous GTPase. (i) We followed exactly the same purification procedure through ion-exchange and gel filtration as we normally use (8) for purifying SpoIIAA, but starting with an extract from a control *Escherichia coli* strain (BL-21) containing no plasmid clone, and detected no GTPase activity. (ii) N-terminal sequencing showed that no protein contaminant could be detected in our preparation. (iii) The GTPase activity corresponded with the peak of elution of SpoIIAA protein when SpoIIAA was subjected to gel filtration by high-performance liquid chromatography. (iv) A polyclonal antibody raised against SpoIIAA completely inhibited the GTPase activity (Table 1). (v) Preincubation of SpoIIAA with SpoIIAB and ATP (20), followed by purification of the resulting SpoIIAA-phosphate by anion-exchange chromatography, completely destroyed both GTP binding and GTPase activity (Table 1). A SpoIIAA mutant protein in which the site of phosphorylation (Ser-58) had been changed to aspartic acid (8) also showed no GTPase activity (Table 1). However, the Ser-58 residue was not essential for the GTPase activity of SpoIIAA, since a mutant protein in which this Ser had been replaced by an Ala residue (8) could hydrolyze GTP, albeit at a diminished rate (Table 1); the addition of SpoIIAB made no difference to the activity of this mutant, SpoIIAAS58A (Table 1).

TABLE 1. GTPase activities of different forms of SpoIIAA[a]

Protein	GTPase activity (cpm)
None	2,335
SpoIIAA	63,230
SpoIIAB	1,901
SpoIIAA + anti-SpoIIAA Ab[b]	2,306
SpoIIAA-P	2,475
SpoIIAAS58D	2,262
SpoIIAAS58A	14,681
SpoIIAAS58A + anti-SpoIIAA Ab	2,822
SpoIIAAS58A + SpoIIAB	13,942

[a] Protein (5 μM) was incubated with 100 μM GTP, and the GTPase activity was measured as described in the text.
[b] Ab, antibody.

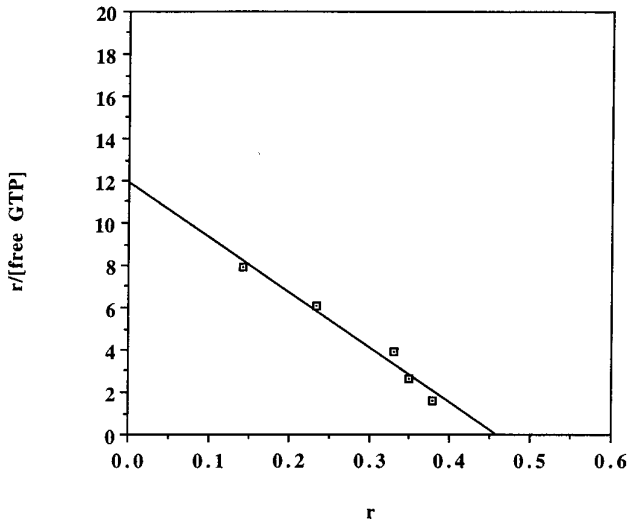

FIG. 2. Scatchard plot of the data obtained by studying the binding of 100 nM SpoIIAA to different concentrations of GTP. In this figure, r = [bound GTP]/[total protein].

Some biochemical features of the GTPase activity of SpoIIAA. (i) Effect of Mg^{2+}. Hydrolysis of GTP by SpoIIAA did not need addition of Mg^{2+} to the reaction mixture, and in fact concentrations of Mg^{2+} above 0.5 mM were inhibitory (Fig. 3). On the other hand, the presence of 20 mM EDTA inhibited GTPase activity by 35% (results not shown). These results suggest that the GTPase activity of SpoIIAA requires very low concentrations of Mg^{2+}; these may have been supplied in our earlier experiments either by copurification of SpoIIAA with divalent cations or by contaminating cations in the GTP. We note that an inhibitory effect of Mg^{2+} on nucleotide exchange by p21$^{N\text{-}ras}$ has been reported (15).

(ii) Effect of nucleoside diphosphate. GDP has relatively little effect on the GTPase activity of SpoIIAA, with a 10-fold excess of GDP in the reaction mixture decreasing the GTPase activity by only 15% (results not shown). A similar effect was observed when ADP was used as a competitor of the ATPase activity of the protein. These results were consistent with the results of the binding experiments in which the SpoIIAA protein showed a very low affinity for GDP or ADP (see above).

(iii) Effect of protein concentration. Although increasing the concentration of SpoIIAA in the GTPase reaction mixture increased the amount of product, it diminished the specific activity of the protein (results not shown). This fact suggests

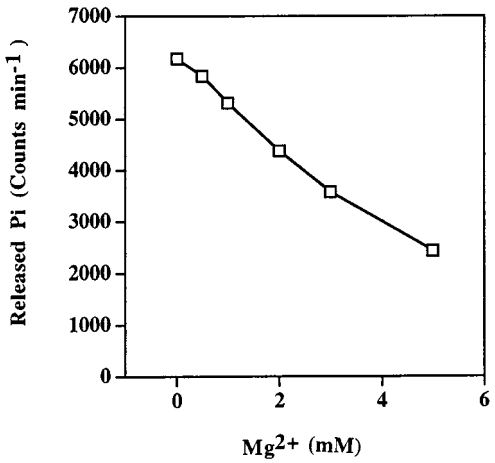

FIG. 3. Effect of Mg^{2+} on the GTPase activity of SpoIIAA. SpoIIAA (5 μM) was incubated with 100 μM GTP for 2 h in a buffer similar to buffer B but containing different concentrations of $MgCl_2$.

the formation of some inactive aggregate of the protein that cannot bind or hydrolyze nucleotides.

Biological significance of GTP binding by SpoIIAA. Since most regulatory GTP-binding proteins interact with the cytoplasmic membrane and are involved in signal transduction pathways (4, 5), it is possible to conjecture that SpoIIAA might bind to a protein in the membrane or asymmetric septum and take part in a pathway that regulates some aspect of sporulation. If there is such a pathway, SpoIIAB might also have a significant role, as SpoIIAB can phosphorylate SpoIIAA (20) and we have shown that SpoIIAA-P can neither bind nor hydrolyze nucleotides (Table 1). On the other hand, it is possible that the GTP-binding activity of SpoIIAA is not physiological but simply reflects its ability to accept a phosphate group from an NTP in the reaction normally catalyzed by SpoIIAB. It might then be argued that SpoIIAA and SpoIIAB are usually present as a complex in the cell (1, 8) and that, in the absence of SpoIIAB, SpoIIAA behaves aberrantly in binding GTP. Arguments against considering GTP binding as nonphysiological include the fact that binding is enhanced by the presence of SpoIIAB and the fact that SpoIIAA evidently has a preference for binding and hydrolysis of GTP rather than of ATP.

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REFERENCES

1. **Alper, S., L. Duncan, and R. Losick.** 1994. An adenosine nucleotide switch controlling the activity of a cell type-specific transcription factor in *B. subtilis*. Cell **77:**195–205.
2. **Bayley, H., and J. R. Knowles.** 1977. Photoaffinity labeling. Methods Enzymol. **46:**69–91.
3. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature (London) **349:**117–127.
4. **Bourne, H. R., and L. T. Williams.** 1994. Cell regulation. Curr. Opin. Cell. Biol. **6:**161–162.
5. **Chant, J., and L. Stowers.** 1995. GTPase cascades, choreographing cellular behavior: movement, morphogenesis, and more. Cell **81:**1–4.
6. **de Boer, P. A. J., R. E. Crossley, A. R. Hand, and L. I. Rothfield.** 1991. The MinD protein is a membrane ATPase required for the correct placement of the Escherichia coli division site. EMBO J. **10:**4371–4380.
7. **Dever, T. E., M. J. Glynias, and W. C. Merrick.** 1987. GTP-binding domain: three consensus sequence elements with distinct spacing. Proc. Natl. Acad. Sci. USA **84:**1814–1818.
8. **Diederich, B., J. F. Wilkinson, T. Magnin, S. M. A. Najafi, J. Errington, and M. D. Yudkin.** 1994. Role of interactions between SpoIIAA and SpoIIAB in regulating cell-specific transcription factor σ^F of *Bacillus subtilis*. Genes Dev. **8:**2653–2663.
9. **Duncan, L., S. Alper, and R. Losick.** 1994. Establishment of cell type specific gene transcription during sporulation in *Bacillus subtilis*. Curr. Opin. Genet. Dev. **4:**630–636.
10. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
11. **Freese, E., J. M. Lopez, and E. B. Freese.** 1979. Initiation of bacterial and yeast sporulation by partial deprivation of guanine nucleotides, p. 129–143. *In* D. Richter and G. Koch (ed.), Regulation of macromolecular synthesis by low molecular weight mediators. Academic Press, New York.
12. **Freissmuth, M., P. J. Casey, and A. G. Gilman.** 1989. G proteins control diverse pathways of transmembrane signaling. FASEB J. **3:**2125–2131.
13. **Gibbs, J. B., I. S. Sigal, M. Poe, and E. M. Scolnick.** 1984. Intrinsic GTPase activity distinguishes normal and oncogenic *ras* p21 molecules. Proc. Natl. Acad. Sci. USA **81:**5704–5708.
14. **Graziano, M. P., and A. G. Gilman.** 1989. Synthesis in *Escherichia coli* of GTPase-deficient mutants of Gsα. J. Biol. Chem. **264:**15475–15482.
15. **Hall, A., and A. J. Self.** 1986. The effect of Mg^{2+} on the guanine nucleotide exchange rate of $p21^{N\text{-}ras}$. J. Biol. Chem. **261:**10963–10965.
16. **Kang Dai, A. M., and J. Lutkenhaus.** 1993. *Escherichia coli* cell division protein FtsZ is a guanine nucleotide binding protein. Proc. Natl. Acad. Sci. USA **90:**1053–1057.
17. **Kaziro, Y.** 1978. The role of guanosine 5′-triphosphate in polypeptide chain elongation. Biochim. Biophys. Acta **505:**95–127.
18. **Lopez, J. M., A. Dromerick, and E. Freese.** 1981. Response of guanosine 5′-triphosphate concentration to nutritional changes and its significance for *Bacillus subtilis* sporulation. J. Bacteriol. **146:**605–613.
19. **Losick, R., and P. Stragier.** 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. Nature (London) **355:** 601–604.
20. **Min, K.-T., C. M. Hilditch, B. Diederich, J. Errington, and M. D. Yudkin.** 1993. σ^F, the first compartment-specific transcription factor of *B. subtilis*, is regulated by an anti-σ factor that is also a protein kinase. Cell **74:**735–742.
21. **Mitchell, C., and J. C. Vary.** 1989. Proteins that interact with GTP during sporulation of *Bacillus subtilis*. J. Bacteriol. **171:**2915–2918.
22. **Price, N. C., and R. A. Dwek.** 1974. Principles and problems in physical chemistry for biochemists, p. 30–39. Oxford University Press, Oxford.
23. **RayChaudhuri, D., and J. T. Park.** 1992. *Escherichia coli* cell-division gene *ftsZ* encodes a novel GTP-binding protein. Nature (London) **359:**251–254.
24. **Schmidt, R., P. Margolis, L. Duncan, R. Coppolecchia, C. P. Moran, and R. Losick.** 1990. Control of developmental transcription factor σ^F by sporulation regulatory proteins SpoIIAA and SpoIIAB in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **87:**9221–9225.
25. **Schweins, T., and A. Wittinghofer.** 1994. GTP-binding proteins: structures, interactions and relationships. Curr. Biol. **4:**547–550.
26. **Sonenshein, A. L.** 1989. Metabolic regulation of sporulation and other stationary-phase phenomena, p. 109–130. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development: a structural and functional analysis of sporulation and germination. American Society for Microbiology, Washington, D.C.
27. **Temeles, G. L., J. B. Gibbs, J. S. D'Alonzo, I. S. Sigal, and E. M. Scolnick.** 1985. Yeast and mammalian *ras* proteins have conserved biochemical properties. Nature (London) **313:**700–703.
28. **Trahey, M., and F. McCormick.** 1987. A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science **238:**542–545.
29. **Vasantha, N., and E. Freese.** 1980. Enzyme changes during *Bacillus subtilis* sporulation caused by deprivation of guanine nucleotides. J. Bacteriol. **144:** 1119–1125.
30. **Welsh, K. M., K. A. Trach, C. Folger, and J. A. Hoch.** 1994. Biochemical characterization of the essential GTP-binding protein Obg of *Bacillus subtilis*. J. Bacteriol. **176:**7161–7168.