Biochemical Characterization of the Essential GTP-Binding Protein Obg of *Bacillus subtilis*†

KATHERINE M. WELSH,* KATHLEEN A. TRACH, CORRADO FOLGER, AND JAMES A. HOCH

Division of Cellular Biology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037

An essential guanine nucleotide-binding protein, Obg, of *Bacillus subtilis* has been characterized with respect to its enzymatic activity for GTP. The protein was seen to hydrolyze GTP with a K_m of 5.4 μM and a k_{cat} of 0.0061 min^{-1} at 37°C. GDP was a competitive inhibitor of this hydrolysis, with an inhibition constant of 1.7 μM at 37°C. The dissociation constant for GDP from the Obg protein was 0.5 μM at 4°C and was estimated to be 1.3 μM at 37°C. Approximately 80% of the purified protein was capable of binding GDP. In addition to hydrolysis of GTP, Obg was seen to autophosphorylate with this substrate. Subsequent release of the covalent phosphate proceeds at too slow a rate to account for the overall rate of GTP hydrolysis, indicating that in vitro hydrolysis does not proceed via the observed phosphoamidate intermediate. It was speculated that the phosphorylated form of the enzyme may represent either a switched-on or a switched-off configuration, either of which may be normally induced by an effector molecule. This enzyme from a temperature-sensitive mutant of Obg did not show significantly altered GTPase activity at the nonpermissive temperature.

Guanine nucleotide-binding proteins as components of signal transduction pathways in eucaryotes have been recognized for many years (15). Only recently has it been discovered that procaryotes contain essential guanine nucleotide-binding proteins which hydrolyze GTP and which may be involved in signal transduction. These include Era (1) of *Escherichia coli*, FtsZ (19, 30) and Ffh (11, 25) of both *E. coli* and *Bacillus subtilis*, and Obg (29) of *B. subtilis*. *obg* was initially identified as a gene downstream of the stage 0 sporulation gene *spo0B* (9, 29). Transcriptional analysis of this operon revealed that *spo0B* and *obg* are cotranscribed. All attempts to inactivate Obg have resulted in loss of viability. While a null mutant of *obg* has not been found, Kok et al. (16) have successfully generated a temperature-sensitive mutant of *obg*. The two mutations in this Obg(Ts) protein are both localized in the N-terminal region of the protein, a region with homology to collagen.

Although Obg was known to be a guanine nucleotide-binding protein, little else was known of its enzymatic capabilities. The enzymatic activities involving GTP of Obg and Obg(Ts) have been examined in this study. The primary activity followed was GTP hydrolysis, including the dependence of this activity on both time of incubation and concentration of Obg and the inhibition of this activity by GDP. An additional activity concerning the autophosphorylation of the protein with [γ-^{32}P]GTP, as well as the subsequent release of the phosphate moiety from the covalently modified enzyme, was also examined. The chemical nature of the covalent modification was determined.

MATERIALS AND METHODS

Bacterial strain. *E. coli* DH5α (F$^-$ *endA1 hsdR17 supE44 thi-1* λ$^-$ *relA1 gyrA96 recA1* φ80*dlacZ*ΔM15) competent cells

were purchased from Bethesda Research Laboratories, Inc. These cells were transformed with pJH4604, which contains the *obg* gene under expression control of a *tac* promoter. Transformed cells were grown at 37°C in Luria broth ([per liter] 10 g of NaCl–10 g of Bacto Tryptone [Difco]–5 g of yeast extract [Difco]) containing 200 μg of ampicillin (Sigma) per ml. These cells were induced at an optical density at 600 nm of 0.5 to 1.0 with 2 mM isopropyl-β-D-thiogalactoside (Diagnostic Chemicals) and harvested by centrifugation at 6,000 × *g* for 15 min after an additional 2 h of growth. Cell pellets were stored at −20°C. In the case of Obg(Ts), DH5α was transformed with pJH4223, which placed *obg*(Ts) under a *tac* promoter. In this case, cells were grown at 30°C.

Reagents. GTP was from Calbiochem. [γ-^{32}P]GTP (6,000 Ci/mmol) and [8,5′-^{3}H]GDP (11.5 Ci/mmol) were supplied by New England Nuclear. IBF Biotechnics was the source of DEAE Trisacryl LS, while Bio-Rad supplied Affi-Gel blue and hydroxylapatite. Pharmacia supplied S200 resin. Activated charcoal, phenylmethylsulfonyl fluoride, and GDP were from Sigma. Proteins used to standardize the S200 column were from Sigma and Bio-Rad. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein standards were from Bio-Rad.

SDS-PAGE. SDS polyacrylamide slab gels (17) were either 12% (acrylamide-bisacrylamide at 29:1) or 15% running gel (15 by 8 cm) with a 5% stacking gel (15 by 1 cm). Samples were prepared by addition of 0.3 eq of tracking dye (167 mM Tris at pH 8.5, 6.7% SDS, 33% glycerol, 0.033% bromophenol blue, 7.3 M β-mercaptoethanol) to 1 eq of Obg (10 μg) or an Obg reaction mixture containing 10 μg of Obg. Unphosphorylated samples were heated at 100°C and run at a constant 200 V until the bromophenol blue reached the bottom of the gel, and the gel was stained with Coomassie blue. Phosphorylated samples were loaded to the gel without heating and run at a constant 25 mA until the bromophenol blue had run 80% of the length of the running gel. The gel was cut just above the dye line to remove any radioactivity associated with GTP or P$_i$. The gel was rinsed briefly in water and either exposed to X-ray film (X-Omat AR5) for ~16 h at −70°C or placed in a phosphoimager cassette and kept for ~16 h at room temperature.

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* Corresponding author. Mailing address: Division of Cellular Biology, Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10666 N. Torrey Pines Rd., La Jolla, CA 92037. Phone: (619) 554-3696.
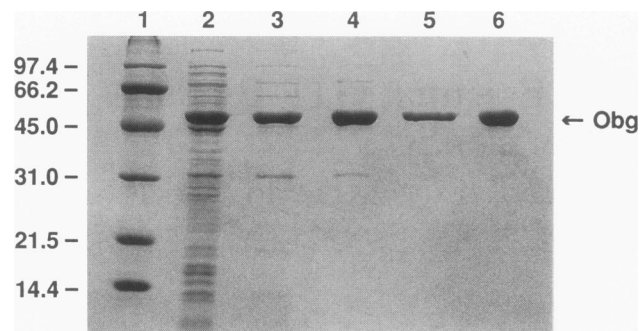
FIG. 1. SDS gel of Obg purification. Lane 1, molecular weight standards (weights, in thousands, indicated at left); lane 2, lysate; lane 3, pool DEAE Trisacryl column; lane 4, pool hydroxylapatite column; lane 5, pool Affi-Gel blue column; lane 6, final concentrate.

Protein determination. Protein was determined by the Bradford method (5) with the Bio-Rad protein assay solution with bovine serum albumin as standard.

Purification. All procedures were performed at 4°C. Frozen cell pellets (induced DH5α with pJH4604 or pJH4223 for the temperature-sensitive mutant) from 4 liters of culture were resuspended in 100 ml of 50 mM Tris at pH 7.5–1 mM EDTA (buffer A) supplemented with 1 mM phenylmethylsulfonyl fluoride. Following sonication, the crude lysate was clarified by centrifugation at 27,000 × g for 30 min in an SS34 rotor. All columns were preequilibrated with buffer A. The supernatant (Fig. 1, lane 2) was applied to a DEAE Trisacryl column (2.5 by 9 cm), washed with 100 ml of buffer A, and eluted with a 1-liter linear gradient of buffer A to buffer A plus 0.5 M KCl. Peak Obg fractions of this and subsequent columns were determined by SDS-PAGE. The relevant fractions were pooled (Fig. 1, lane 3) and applied to a hydroxylapatite column (1.5 by 13 cm). The column was washed with 100 ml of buffer A and eluted with a 250-ml linear gradient of buffer A to 100 mM KP_i at pH 7.5–1 mM EDTA. Obg-containing fractions were pooled (Fig. 1, lane 4), applied to an Affi-Gel blue column (2.5 by 9 cm), and eluted with a 1-liter gradient of buffer A to buffer A containing 1.0 M KCl. The appropriate fractions were pooled (Fig. 1, lane 5), concentrated on a 2-ml hydroxylapatite column, and eluted with 100 mM KP_i at pH 7.5–1 mM EDTA–20% glycerol. The concentrated protein (~3.5 mg/ml) was stored in 0.5-ml aliquots at −20°C. Prior to use, Obg was equilibrated on a Pharmacia Superdex 75 16/60 column with 50 mM Tris at pH 7.5–100 mM KCl (buffer B) and concentrated with an Amicon Centriprep 10 at 1,500 × g.

Determination of native molecular weight. Obg (1.7 mg/ml) and Obg(Ts) (3.3 mg/ml) were separately chromatographed on an S200 column (2.5 by 84 cm) in buffer B. The column was standardized with cytochrome c (12.4 kDa, 4 mg/ml), equine myoglobin (17 kDa, 5 mg/ml), carbonic anhydrase (29 kDa, 4.5 mg/ml), chicken ovalbumin (44 kDa, 10 mg/ml), bovine serum albumin (66 kDa, 10 mg/ml), yeast alcohol dehydrogenase (150 kDa, 7.5 mg/ml), bovine γ-globin (158 kDa, 10 mg/ml), and thyroglobulin (670 kDa, 10 mg/ml).

Hydrolysis of GTP. Obg was analyzed for its ability to hydrolyze [γ-^{32}P]GTP to the corresponding GDP plus P_i. Linearity as a function of time was established at 37°C with Obg at 0.7 μM and GTP at 10 μM. Assays were followed over 4 h under standard conditions, except that KCl was present at 100 mM. Standard assay conditions were 50 mM Tris at pH 8.5, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 200 mM KCl, 10% glycerol, Obg or Obg(Ts) at 0.8 to 1.7 μM, and [γ-^{32}P]GTP at 2 (12.6 ×

10^6 cpm/nmol) to 40 μM (6.3 × 10^5 cpm/nmol). Reactions with Obg were run at 37°C for 3 h, while those with Obg(Ts) were run at 37°C for 20 min. The observed rate is given in picomoles per minute-milligram. Samples were quenched with a slurry of charcoal in 1 mM KP_i at pH 7.5, which would selectively bind GTP and GDP while leaving the released P_i in solution. The charcoal was pelleted by centrifugation, and the free phosphate was determined by Cerenkov counting of the supernatant.

Given the weak homology with collagen of the N-terminal domain of Obg (29) and the apparent monomeric structure, it was of interest to see whether the rate as a function of enzyme concentration would suggest a more complex quaternary structure. The enzyme was assayed as described above except that the GTP was 10 μM (4.2 × 10^6 cpm/nmol) and Obg was varied from 0.21 to 4.2 μM in 0.21 μM increments. Reactions were quenched with charcoal at times which varied from 1/2 to 3 h depending upon Obg concentration in the assay. The extent of turnover was controlled at ≤8%.

Inhibition of GTP hydrolysis by GDP. Reaction conditions for inhibition of GTP hydrolysis by GDP were the same as those described for GTP hydrolysis except that 0, 2, 4, or 8 μM GDP was present in the reactions. Samples were quenched, the charcoal was pelleted by centrifugation, and free P_i was determined by Cerenkov counting of the supernatant. The resultant data were fit to an equation describing competitive inhibition of a single substrate enzymatic reaction.

Binding of GDP. Equilibrium dialysis at 4°C was utilized to determine the dissociation constant of GDP for Obg. Reactions were run in duplicate in a dialysis wheel with 0.1-ml chambers on each side separated by a dialysis membrane. Initial conditions of the nonenzyme side were 50 mM Tris at pH 8.5, 100 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and seven GDP concentrations (2.56 × 10^5 cpm/nmol) ranging from 4.8 to 0.48 μM. Initial conditions of the enzyme side were 50 mM Tris at pH 8.5, 100 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, and Obg at 1.5 μM. The dialysis cells were rotated overnight. Samples were removed from each chamber, and an aliquot was counted by liquid scintillation in NEN BIOFLUOR to determine the concentration of GDP at equilibrium. Protein concentration was determined in those samples which contained Obg.

Autophosphorylation with GTP. Obg at 10 μM was autophosphorylated with 5 μM [γ-^{32}P]GTP (30 × 10^6 cpm/nmol) in 50 mM Tris at pH 8.5, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 100 mM KCl, and 10% glycerol. Reactions were run at 37°C for 30 min and quenched with tracking dye, and three identical samples were loaded onto an SDS–12% polyacrylamide gel and electrophoresed at 25 mA.

Identification of the type of residue which is autophosphorylated on Obg. After autoradiography, the gel was sliced into three parts, each containing one radioactive Obg band. One slice was saved as a control, one was incubated at 55°C for 1 h in 0.2 N HCl, and a third was incubated at 55°C for 1 h in 1 N NaOH (6). After this treatment, the gel slices were again exposed to X-ray film.

Rate of release of P_i from the Obg phosphoenzyme. Obg (10 μM) or Obg(Ts) (10 μM) was autophosphorylated with 25 μM [γ-^{32}P]GTP (2.4 × 10^6 cpm/nmol) in 50 mM Tris at pH 8.5, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 160 mM KCl, and 10% glycerol in a total volume of 2 ml. The reaction was run at 37°C for 3 h, added to a Pharmacia Superdex 75 column, and eluted with buffer B. Fractions were analyzed for protein concentration and extent of phosphorylation. The appropriate fractions were pooled and concentrated with an Amicon Centricon 30. The phosphorylated Obg protein was incubated at 37°C in 50 mM

Tris at pH 8.5, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 200 mM KCl, 10% glycerol, and either no nucleotide triphosphate or 2.5 mM GTP. As a function of time, 20-μl samples were withdrawn, quenched with 6 μl of tracking dye, and stored at $-20°C$ until the last samples were taken at 50 h. Quenched samples were thawed, loaded to an SDS–15% polyacrylamide gel, and run at 25 mA. The resultant running gel was exposed to a Molecular Dynamics Phospho Screen overnight at room temperature. Data were obtained with a Molecular Dynamics Phosphoimager and analyzed to determine the first-order rate constant of dephosphorylation.

RESULTS

Purification of Obg or Obg(Ts) resulted in a final product which ran at the appropriate subunit molecular mass (47.7 kDa) on SDS gels. The homogeneity of the final product (Fig. 1, lane 6) was greater than 98%. The yield per liter of culture of the purified protein was 5 to 10 mg. When examined for quaternary structure by molecular sieve chromatography, both Obg and Obg(Ts) eluted at a relative molecular weight of 56,000. This value is approximately 12% higher than the subunit molecular weight calculated from the protein's primary sequence. This difference may reflect an imperfect spherical shape of the protein, and it is believed that Obg is, indeed, monomeric. Protein autophosphorylated in vitro also eluted at a relative molecular weight of 56,000, indicating that this phosphorylation does not change the quaternary structure of Obg.

GTPase activity of Obg. Analysis of the primary sequence of Obg revealed a central portion of the protein which contained sequences identified with guanine nucleotide-binding sites in G proteins, elongation factors of *E. coli*, mammalian Ras, and procaryotic proteins such as Era (29). These representatives of a superfamily of proteins are known to both bind and hydrolyze GTP (3, 4). Photoactivation of GTP in the presence of Obg resulted in a covalent modification of Obg which was stable to acid precipitation, heat, and SDS-PAGE (29). While this evidence was sufficient to indicate binding of GTP to Obg,

TABLE 1. Assay requirements for Obg

Variable component	Concn of component (mM)	% Relative activity
None[a]	0	100
EDTA	0	99
KCl	100	85
KCl	0	20
Mg^{2+}	0	<3

[a] Standard assay conditions are given in Materials and Methods; [GTP] = 100 μM.

it could not address the issue of Obg's enzymatic activity with respect to GTP.

It was therefore of interest to examine directly Obg's ability to hydrolyze GTP. As a first approach, GTP radiolabelled in either the α or γ phosphate was incubated with Obg and samples of the reaction were applied to a polyethyleneimine cellulose thin-layer plate. The plate was developed under conditions which would separate GMP, GDP, GTP, and P_i (24). Following incubation with Obg, GTP labelled in the γ position was found to release labelled P_i. GTP labelled in the α position generated labelled GDP on hydrolysis by Obg. This established that Obg catalyzed the hydrolysis of GTP between the β and γ phosphates, generating GDP and P_i (data not shown). The position of hydrolysis having been established, optimal conditions for this hydrolysis were determined. It can be seen from Table 1 that the divalent metal ion, Mg^{2+}, was essential for hydrolysis and that KCl enhanced the hydrolysis rate. ATP could not substitute as a substrate for hydrolysis.

Quantitation of GTPase activity was accomplished by separation of GTP and GDP from P_i by the selective binding of the nucleotides to charcoal. Other procaryotic GTPases have shown a kinetic lag in the hydrolysis of GTP as a function of time (22). There has also been evidence for cooperativity (22, 30) when GTP hydrolysis has been examined as a function of protein concentration. To exclude these complications with Obg, the hydrolysis of GTP was examined as a function of
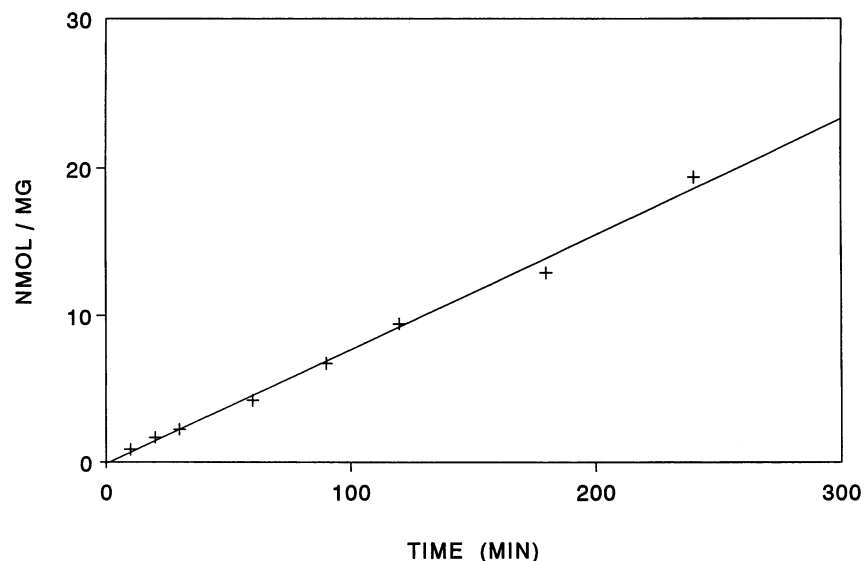

FIG. 2. Linearity of GTP hydrolysis as a function of time. Reactions were run as described in Materials and Methods with GTP present at 10 μM and Obg present at 0.7 μM. Samples were taken over a 4-h period.
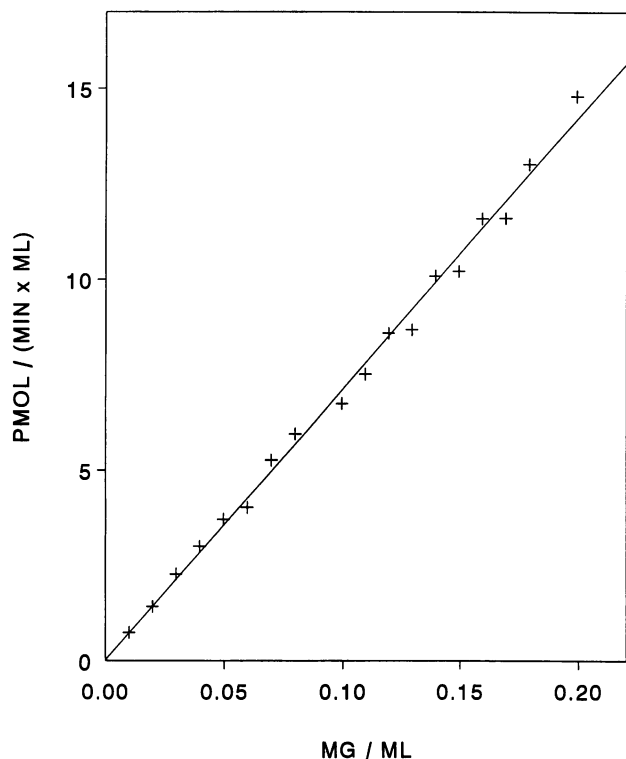
FIG. 3. Linearity of GTP hydrolysis as a function of protein concentration. GTP was present at 10 μM under standard hydrolysis conditions. The Obg concentration was varied from 0.21 to 4.2 μM. The time of each reaction was varied to control the turnover of substrate at ≤8%.

Fig. 3 (78 and 73 pmol/min · mg, respectively) are consistent with the rate later observed when a substrate concentration curve was examined.

The hydrolysis of GTP by Obg was observed to be saturable and could be represented by the standard equation for a single substrate enzyme reaction. The observed K_m^{GTP} = 5.4 μM, while the maximum velocity is 127 pmol/min · mg, which corresponds to a k_{cat} of 0.0061 min^{-1}. These data are shown in Fig. 4 by the line determined in the absence of GDP.

Inhibition of GTP hydrolysis by GDP. GMP, GDP, and several analogs of GTP were examined to identify an inhibitor of GTP hydrolysis by Obg. The initial screen indicated that GDP was the best inhibitor of those tested. A more extensive analysis of GDP inhibition was subsequently undertaken, and the results are presented in Fig. 4. Apparent in the figure is the characteristic Lineweaver-Burk pattern of competitive inhibition, in which the rate at saturating GTP concentration converges on V_{max}. The data can be fit to the equation for competitive inhibition of a single substrate enzymatic reaction in which we can identify K_m^{GTP} the apparent = $K_m^{GTP}\{1 + ([GDP]/K_i^{GDP})\}$. Replotting the observed apparent K_m^{GTP}, at each concentration of GDP against the GDP concentration allows the K_i^{GDP} to be determined from the quotient of the intercept to the slope of the resulting line. This yields a K_i^{GDP} of 1.7 μM.

Determination of the dissociation constant for GDP. In order to confirm the observed K_i^{GDP} and to establish a measure of the fraction of active protein present in the purification, equilibrium dialysis with ^{3}H-labelled GDP and Obg was undertaken. The results of this experiment at 4°C are presented as a Scatchard (26) plot in Fig. 5 and can be described by the Scatchard equation for a single dissociation constant in which the fraction bound = nanomoles of bound GDP/nanomoles of Obg and n is equivalent to the number of available sites on the protein. The K_d^{GDP} derived from the slope of this line is 0.5 μM, while the abscissa intercept indicates that 0.78 mol of GDP is bound per mol of Obg. Sizing of Obg by molecular sieve chromatography had indicated that the protein is monomeric, so that approximately 80% of the
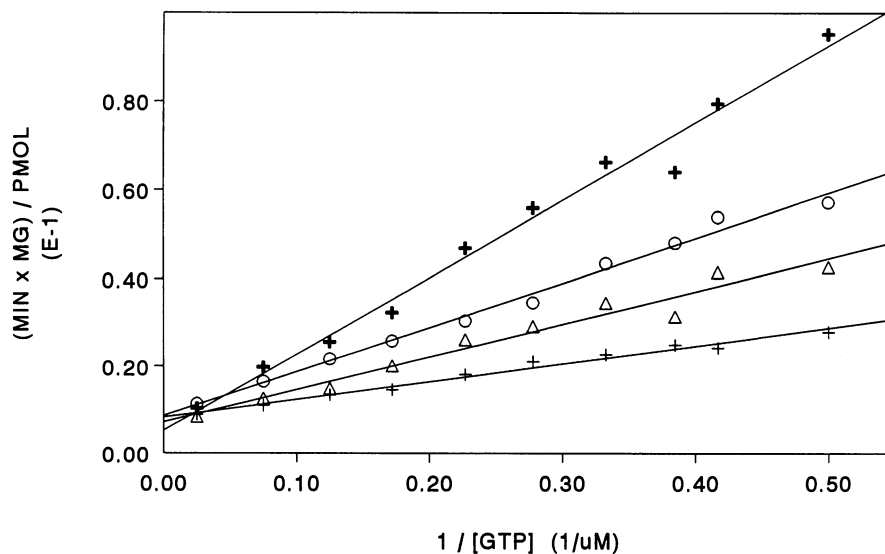
reaction time and protein concentration. Hydrolysis of GTP by Obg was found to be linear with respect to both time (Fig. 2) and protein concentration (Fig. 3). The hydrolysis rates at 10 μM GTP which can be calculated from the slopes of Fig. 2 and

FIG. 4. Inhibition of Obg's hydrolysis of GTP by the product GDP. Reactions were run as described in Materials and Methods. GDP was present at 0 μM (+), 2 μM (△), 4 μM (○), or 8 μM (+). Data are presented as a Lineweaver-Burk plot.
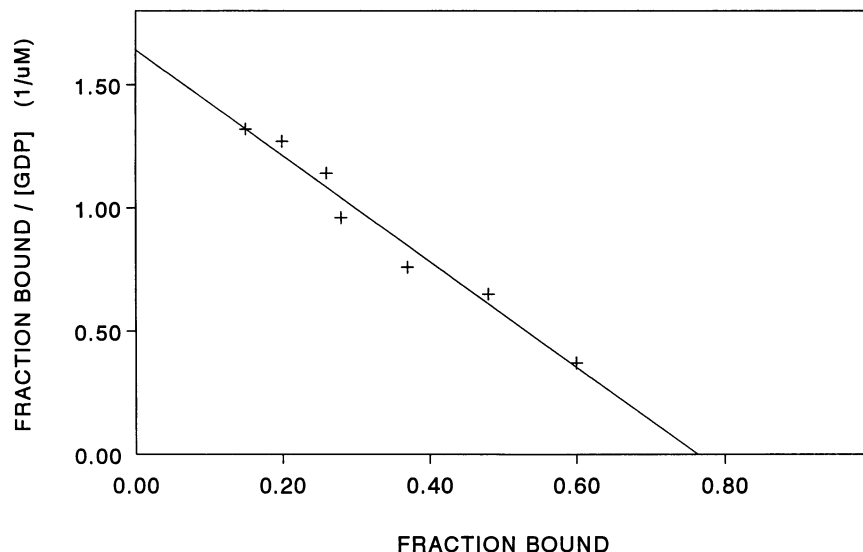
FIG. 5. Scatchard plot of GDP binding to Obg. Equilibrium dialysis was run as described in Materials and Methods.

purified protein can be considered to be capable of binding GTP.

To estimate the K_d^{GDP} at 37°C, equilibrium dialysis was repeated at 25°C. In this case, the observed K_d^{GDP} was 0.93 μM. With the dissociation constants at 4 and 25°C, the van't Hoff equation was utilized to estimate the value at 37°C. Plotting $-\ln K_d^{GDP}$ versus the reciprocal of temperature in kelvins generated a line from which the K_d^{GDP} value of 1.3 μM at 37°C can be estimated. This value is very close to the K_i^{GDP} of 1.7 μM determined from the inhibition kinetics.

Autophosphorylation of Obg. It has been observed with certain oncogenic (14) forms of Ras that the protein is autophosphorylated by GTP. As a consequence, the ability of Obg to autophosphorylate with GTP as a substrate was examined and Obg was found to be covalently modified. The nature of the phosphorylated residue was investigated by observation of the stability of the protein-phosphate bond in acid or base (6). Figure 6A presents the autoradiograph of three equivalent phosphorylated Obg samples following electrophoresis on SDS-polyacrylamide gels. Three slices of this gel were then examined for acid-base stability as described in Materials and Methods. Following acid or base treatment, the gel slices were again autoradiographed. It is obvious from Fig. 6B that the in vitro-phosphorylated form of the protein is stable to base (lane 1) but labile in acid (lane 2). This result implicates the modification of a nitrogen group on the protein, generating a phosphoamidate. ATP could not substitute for GTP in the in vitro autophosphorylation reaction.

It was necessary to examine this phosphorylated form of Obg in greater detail to determine if the hydrolysis of GTP proceeded by a covalent phosphoenzyme intermediate. Obg-P was prepared and separated from excess GTP by chromatography through a molecular sieve column. It was noted that the eluted radiolabelled phosphoprotein represented only 0.002 eq of the total protein. With this protein, the off-rate of labelled phosphate under standard GTPase assay conditions was examined. The release of phosphate was followed over 50 h at 37°C and seen to be first order (Fig. 7). The rate of release of this covalently bound phosphate is equivalent to $0.693/(t_{1/2})$, where $t_{1/2}$ is defined as the time required to remove 50% of the phosphate. This rate is equivalent to 0.00036 min^{-1}. This

off-rate, which is equivalent to the hydrolysis rate of covalently bound phosphate, is only 6% of the observed k_{cat} of GTP hydrolysis and is not consistent with the covalent phosphate lying along the reaction path of this enzyme. While the data in Fig. 7 were obtained in the absence of GTP, only minimal (<15%) decrease in the half-life was observed in the presence of 2.5 mM GTP, a result which also suggests that Obg-P is not on the reaction pathway.

Effect of temperature on GTPase activity of Obg and Obg(Ts). A Ts mutant of Obg [Obg(Ts)] has recently been isolated (16). This protein has been purified by the same procedure as that which was used for the wild-type protein. The protein is observed to be monomeric both before and after in vitro autophosphorylation. Small differences in the kinetic
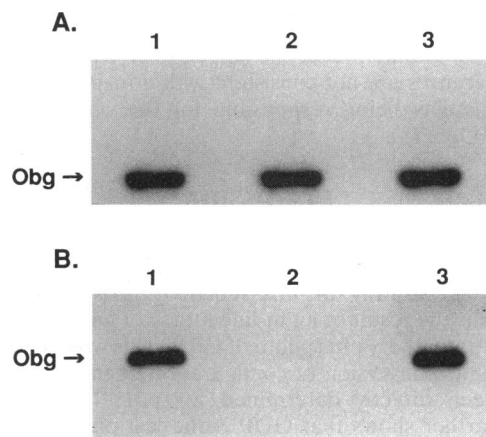

FIG. 6. Acid-base stability of phosphorylated Obg. Obg was autophosphorylated as described in Materials and Methods. (A) Three equivalent samples of Obg-P electrophoresed on an SDS-12% polyacrylamide gel and autoradiographed. (B) The samples from panel A were sliced into three fragments. Lane 1 was treated with 1.0 N NaOH, lane 2 was treated with 0.2 N HCl, and lane 3 represents an untreated control. Following treatment, the three fragments were again autoradiographed.
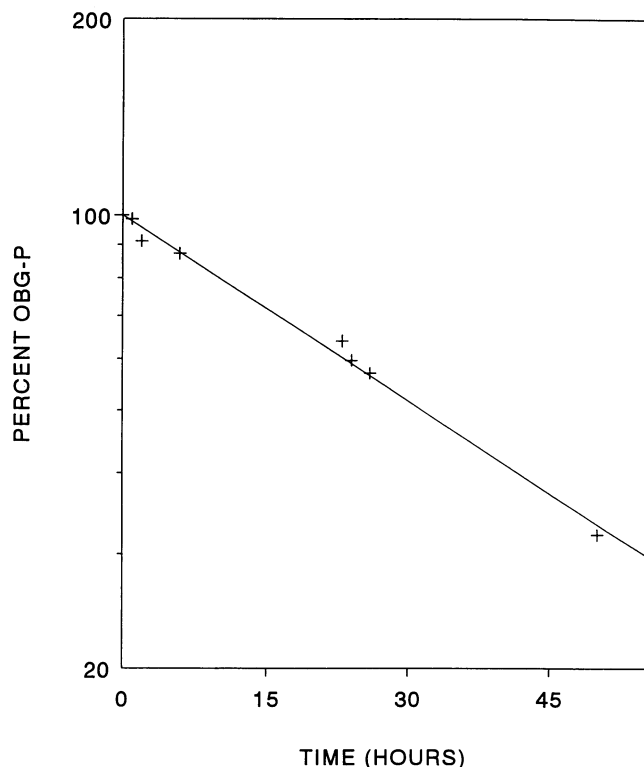
FIG. 7. Release of P_i from the covalent Obg-P complex. Experimental conditions are described in Materials and Methods. The first-order rate constant was derived from the time required to reduce Obg-P by 50%.

FIG. 8. Kinetic scheme of Obg. E represents one monomer of the Obg protein. P represents inorganic phosphate. A dash denotes covalency.

parameters between Obg(Ts) and Obg have been noted. For Obg(Ts), the $k_{cat} = 0.015$ min^{-1}, while the $K_m^{GTP} = 2.3$ μM. Analysis of both Obg(Ts) and Obg at 45°C instead of 37°C revealed only small changes in k_{cat} and K_m, consistent with results which might be expected from an increase in temperature of 8°C. When autophosphorylated in vitro, 0.008 eq of phosphate was present per eq of protein. The observed off-rate of this radiolabelled phosphate was seen to be 0.00045 min^{-1}. This off-rate is only 3% of the k_{cat} of GTP hydrolysis. These small differences are not consistent with a major effect on the GTPase activity being responsible for the observed in vivo effects of Obg(Ts).

DISCUSSION

A kinetic scheme which represents Obg hydrolysis of GTP is presented in Fig. 8. In this scheme, the bold arrows represent the primary pathway of this reaction. It is believed that hydrolysis is the result of an in-line attack of an activated H_2O molecule upon the γ phosphate of GTP. This would result in a stereochemical inversion of configuration of the γ phosphate, as has been directly determined for p21$^{c-Ha-ras}$ (10). The scheme further shows that GDP is the last product released, which is consistent with the observed competitive inhibition of GDP for the substrate GTP, implying that both nucleotides interact with the same form of the free enzyme.

The light arrows in the scheme represent what is believed to be a minor reaction catalyzed by Obg in vitro which generates a covalently phosphorylated form of the enzyme. Only low, less-than-1% phosphorylation of the protein has been observed in vitro. If this phosphoenzyme form was on the overall
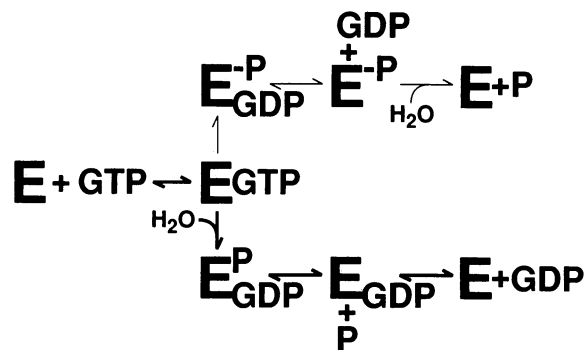
reaction pathway, the release of radiolabelled covalent phosphate from the enzyme would have to be at least as fast as the observed k_{cat} of the overall reaction. However, the observed off-rate of covalently bound phosphate from Obg is less than 10% of the k_{cat} for GTP hydrolysis. Consequently, the significant in vitro hydrolysis of GTP cannot proceed via this phosphoenzyme intermediate.

The acid-base stability of this phosphoenzyme establishes that a phosphoamidate has been formed. On the basis of probable structure homology with the Ras protein (8), it is believed that the modified residue is His-189 of Obg. This equivalent position in the Ras protein lies within loop 2, a region of the protein which is seen to move further into the substrate pocket on binding GTP (21). Recently, Sood et al. (28) have shown that Era from E. coli can be autophosphorylated by GTP, generating both phosphoserine and phosphothreonine residues. Sequence localization of the modified residues with respect to the Ras structure placed them within the equivalent of loop 2 of Ras. For Ras, loop 2 is known to be involved in the interaction of the protein with GAP (31).

It is unclear at this time whether Obg is phosphorylated in vivo. In vivo phosphorylation has been observed for both Era (28) and certain mutants of p21^{Ha-ras} (14). In the case of p21 from Harvey murine sarcoma virus (27), the radiolabelled peptides resulting from both in vivo and in vitro labelling are identical, suggesting that phosphorylation of this protein is an autophosphorylation both in vitro and in vivo. It is possible that Obg is extensively phosphorylated in vivo, thus limiting the in vitro phosphorylation to less than 1%. If this were the case, phosphorylated Obg would be capable of both binding and hydrolyzing GTP. Alternatively, the protein as isolated from E. coli may be poorly phosphorylated and the observed low extent of in vitro autophosphorylation may be the result of the absence of a B. subtilis effector molecule. In this case, it will not be clear whether the phosphorylated protein remains active in binding and/or hydrolyzing GTP.

It is unknown whether the phosphorylated form of Obg has any signal-transducing activity, although it is believed that Obg, as a member of the superfamily of guanine nucleotide-binding proteins, functions in vivo in some (at this time undefined) signalling pathway. It is interesting to speculate that the phosphorylated form of the molecule might represent either a switched-on or a switched-off conformation (3, 4) of Obg that may be normally induced by some effector molecule. In the absence of such a molecule in vitro, only a small portion of the protein may be found in this conformation. Alternatively, the phosphorylation of Obg might be required to generate the

proper charge distribution or hydrogen-bonding potential for interaction with a select effector molecule or transducing-signal recipient. Phosphorylation does not alter the molecular weight of Obg, indicating that it does not modify the aggregation state of Obg.

Of the procaryotic guanine nucleotide-binding proteins which have been examined in vitro, Era most closely resembles Obg in parameters such as k_{cat}, K_m^{GTP}, and K_d^{GDP} (7). Unlike Ras, both proteins have micromolar binding constants for GDP. In the case of Ras, it has been argued that an aromatic-aromatic interaction between the guanine base and Phe-28 are, in part, responsible for the high affinity which Ras has for both GDP and GTP (31). This phenylalanine residue is not conserved in either Era or Obg.

Both Era (13, 18, 20) and Obg (16) are essential for cellular viability, and both have yielded Ts mutations. In contrast to the Ts Era, both wild-type and Ts forms of Obg are quite similar in kinetic parameters with respect to GTP hydrolysis, at permissive and nonpermissive temperatures in vitro. It has been speculated that in the case of Era, the Ts GTPase activity is responsible for the loss of viability at the nonpermissive temperature (18). This cannot be the case for Obg(Ts). It seems unlikely that the observed in vivo phenotype of the Ts mutant is the result of the slightly higher k_{cat} for GTP hydrolysis. It is generally believed that the members of the GTPase superfamily function by cycling between the GTP-bound on state and the GDP-bound off state (3, 4). In terms of Obg lying on a signal-transducing pathway, this slight activation of Obg(Ts) would shut off the presumed signalling form of the protein more quickly. Such slight activation is trivial compared with the 10^5-fold increase in GTP hydrolysis rate seen for Ras in the presence of GAP (2, 31). It seems more likely that the Ts mutation in Obg(Ts) impairs the ability of this protein to interact with other proteins of the signal transduction pathway.

The rate of GTP hydrolysis by Obg, 0.006 min^{-1}, is similar to that which has been observed for Ras and is much slower than the rates for the α subunit of G proteins, which are in the range of 3 to 5 min^{-1} (15). The rate at which Ras can hydrolyze GTP is greatly enhanced by effector molecules such as GAP (2, 31). Although it can be speculated that a GTPase-activating protein may well function in the signal transduction pathway of Obg, no evidence for such a protein has yet been found.

Bourne et al. (4) have described primary structure sequence motifs (G1 to G5) which are associated with the GTPase superfamily. Analysis of the primary sequence of Obg, deduced from the nucleotide sequence of the gene, revealed a central portion of the protein which contained amino acid motifs characteristic of a guanine nucleotide-binding protein (29). It was later proposed on the basis of the G1 (P-loop) sequence that Obg and several other proteins belonged in a discrete subfamily of guanine nucleotide-binding proteins, all of which are of unknown function (12). Further examination of the amino acid sequences of Obg, *Schizosaccharomyces pombe* GTP1, and the deduced product of an open reading frame of *Halobacterium cutirubrum* reveal a conserved G2 sequence, Y(E/H)FTTL, supporting the argument that these three proteins belong in a distinct subfamily. It has also been suggested that HflX of *E. coli* (23) also belongs in this subfamily. However, to the extent that G1 and G2 are subfamily specific, HflX has probably been misassigned.

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