Biochemical Characterization of Aspartyl Phosphate Phosphatase Interaction with a Phosphorylated Response Regulator and Its Inhibition by a Pentapeptide*

Shu Ishikawa, Leighton Core, and Marta Perego‡

Received for publication, February 1, 2002, and in revised form, March 25, 2002
Published, JBC Papers in Press, March 28, 2002, DOI 10.1074/jbc.M201089200

The RapA and RapB proteins are aspartyl phosphate phosphatases that specifically dephosphorylate the Spo0F–P intermediate response regulator of the phosphorelay signal transduction system for sporulation initiation in Bacillus subtilis. The ~48-kDa His-tag derivative proteins were purified by metal affinity chromatography, and their molecular and biochemical characteristics were studied. RapA and RapB were found to be dimers in solution. Enzymatic activity was strongly dependent upon maintaining reducing conditions during purification and storage. RapA phosphatase activity on Spo0F–P is inhibited in vivo by a pentapeptide generated from the phrA gene. Native gel assays demonstrated that the RapA dimer forms a stable complex with two molecules of Spo0F–P or with its PhrA pentapeptide inhibitor. The pentapeptide was shown to displace Spo0F–P from a preformed complex with RapA. The structural organization of Rap phosphatases in tetratripeptide repeats provides insights on the mechanisms of RapA interaction with its substrate and its inhibitor.

The kinase activities are counteracted by protein phosphatases that respond to environmental and physiological signals antithetical to sporulation (5). The Rap family of phosphatases is comprised of 11 closely related proteins in B. subtilis (6, 7). Among them RapA, RapB, and RapE activities are directed toward the aspartyl phosphate of the single domain response regulator Spo0F of the phosphorelay (6, 8). The cellular activity of Rap phosphatases in sporulation is modulated both through transcription regulation and by inhibition of enzymatic activity. rapA, rapB, and rapE gene transcription is regulated by conditions antithetical to sporulation such as competence to DNA transformation or vegetative growth (6, 8, 9). Inhibition of enzymatic activity involves the production of specific pentapeptide inhibitors through a complex export-import pathway. The pentapeptide inhibitor of RapA is encoded by the rapA-associated phrA gene as a 44-amino acid precursor that, after being exported by the SecA-dependent system and processed to the carboxyl-terminal five-amino acid active inhibitor (ARNQT), is reimported by the oligopeptide permease transport system to act intracellularly (10). Production of the PhrE pentapeptide follows a similar pathway (6). Phr pentapeptides are specifically active against their cognate Rap phosphatase, and their specificity is highly dependent on the amino acid sequence of the pentapeptide and on the presence of the carboxylic acid at the carboxy-terminal end of the peptide (11).

In this report we describe the biochemical characterization of the RapA phosphatase activity and provide the first evidence of complex formation between the enzyme and its substrate Spo0F and/or its inhibitor, the PhrA pentapeptide.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The Escherichia coli strain DH5α was used for plasmid construction and propagation. The E. coli strain BL21(DE3)pLysS (Novagen) was used for Rap protein overexpression. Plasmid pET16b (Novagen) was used for the construction of the RapA and RapB expression systems (8) in E. coli. Plasmid pET20b (Novagen) was used to produce a Spo0F protein fused to six His residues at the carboxyl terminus as described in Tzeng and Hoch (12).

RapA fused to six His residues at the carboxyl terminus was obtained for in vivo studies as follows. A 570-bp fragment containing the 3′ half of the rapA gene was amplified by PCR reaction using at the 5′ end, oligo OL-2 (GTCCGCAGAATCCAATG), which immediately precedes an EcoRI site, and at the 3′ end, oligo RapA 3′ His (GGATCTCTGGAT-TAATGATGATGATGATGATTCTATATACAAACTTCC), which fused six histidine codons and a PetI site. The fragment was cloned in the EcoRI-PetI sites of the integrative vector pJM103 (13), resulting in plasmid pRAH. This plasmid was transformed in the wild-type strain JH642 (trpC2, phe-1), where it recombined with the chromosome by a single crossover event, resulting in strain JHRAH that expresses an active RapA-His₆ protein.

Protein Purification—The E. coli expression strain BL21(DE3)pLysS was freshly transformed with plasmid pET16b-RapA or pET16b-RapB and grown in LB medium containing ampicillin (100 μg/ml) to an A₆₀₀ of 0.7. Protein expression was induced with isopropyl-1-thio-β-D-galacto-
topranoside (final concentration 1 mM), and the cells were incubated for an additional 2 h. The cells were harvested and immediately resuspended in lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol; pH 7.9). Cell disruption was obtained by means of three passages through a French press. After removal of cell debris by ultracentrifugation at 45,000 rpm for 2 h at 18°C, the supernatant was loaded onto a nickel nitritrocetic acid-agarose column (Qiagen). The His-tagged proteins were eluted with a 10–200 mM imidazole linear gradient in lysis buffer. The fractions containing the protein were identified by SDS-PAGE and Coomassie Blue staining, pooled, and dialyzed against the storage buffer (20 mM HEPES (pH 7.0), 0.3 M NaCl, 10 mM imidazole). The column was washed with 10 ml of binding buffer, and protein elution was obtained with binding buffer containing 100 mM NaCl, 10 mM imidazole (pH 7.0), and 10 mM MgCl2. The proteins were concentrated using a Centriprep-30 concentrator (Amicon), frozen in a dry ice/ethanol bath, and stored at −80°C. Purification of Spo0F-His6 tag and KinA was as previously described (12).

**Purification of Spo0F and RapA**—A Spo0F protein modified to contain His6 residues at the carboxyl-terminal end was phosphorylated (50 μM) with KinA (0.5 μM) and [γ-32P]ATP (1 mM specific activity of 0.6 Ci/mmol) in the phosphorolysis buffer (50 mM EPES (pH 8.5), 20 mM MgCl2, 0.1 mM EDTA 5% glycerol) for 2 h at room temperature. The reaction mixture was loaded on a 0.5-Ml column of nickel-bound His-bind resin (Novagen) equilibrated with binding buffer (20 mM Tris-HCl (pH 7.9), 0.3 M NaCl, 10 mM imidazole). The column was washed with 10 ml of binding buffer, and protein elution was obtained with binding buffer containing 150 mM NaCl. Fractions containing radioactivity were dialyzed in 20 mM HEPES, 0.1 mM EDTA, and 10% glycerol (pH 7.0). Aliquots were frozen in a dry ice/ethanol bath and stored at −80°C. The ratio of Spo0F/Spo0F-P in the purified sample, 20/80, was determined by analysis of a 10% Tris-glycine native PAGE with an Ultra Scan XL laser densitometer (Amersham Biosciences). The concentrations of Spo0F/Spo0F-P used throughout this study refer to the fraction of Spo0F-P only.

**Rap Phosphatase Assay**—The basic dephosphorylation assay used to optimize the reaction conditions was carried out in the presence of 10 mM DTT, 0.1 mM EDTA, and 20% glycerol. The buffers tested (MES (pH 6.0–6.5), HEPES (pH 7.0–7.5), TAPS (pH 8.0–9.0), CHES (pH 9.0–10.0), CAPS (pH 10.0–11.0), Bis-Tris propane (pH 6.5–9.5)) were all used at a concentration of 50 mM, and MgCl2 was tested at the concentrations indicated in Fig. 2. RapA (1 μM) was preincubated in the reaction buffer for 10 min at 25°C. Radioactively labeled and purified Spo0F-P (2.5 μM) was added to initiate the reaction. Samples were removed at various intervals, mixed with an equal volume of 2× loading dye buffer, and frozen in a dry ice/ethanol bath. The samples were run on a 15% Tris-Tricine–SDS gel and analyzed by PhosphorImager and ImageQuant software (Molecular Dynamics).

**Native Polyacrylamide Gel Electrophoresis**—Tris–Tricine native PAGE was carried out according to Schaeffer and von Jagow (14) with some modifications. The gels were prepared using a 3% Tris–HCl buffer at pH 8.45 containing 1 mM EDTA. The running buffer contained 0.1 mM Tris–HCl, 0.1% Tricine, and 1 mM EDTA (pH 8.25). Protein molecular weights were determined using molecular weight markers for non-denaturing polyacrylamide gel electrophoresis (Sigma) as standards and the calculations were carried out as described by the supplier's instructions.

**In Vivo and In Vitro Cross-linking**—Strain JHRAH was grown in Schaeffer's sporulation medium (15). Cells were monitored at A600 and harvested 1 h after the transition to stationary phase. Cells were suspended in 50 mM HEPES (pH 7.0), 1.5 mM MgCl2, 50 mM NaCl. The EGS cross-linker (Pierce) resuspended in Me2SO was added to 0.4 mM final concentration. A 30-min incubation at room temperature was allowed before stopping the reaction with 2× glycine, 50 mM Tris-HCl, pH 7.0. Cells were centrifuged and resuspended in lysis buffer (50 mM Tris-HCl (pH 7.0), 1.5 mM MgCl2, 50 mM NaCl, 2 mg/ml lysozyme, 10 μg/ml DNase I, 1 mM phenylmethylsulfonyl fluoride). Lysis was allowed to occur for 15 min at 37°C. Cell debris was removed by centrifugation at 15,000 rpm for 15 min. The lysates were mixed with nickel nitritrocetic acid-agarose (Qiagen) (1 ml of resin/100 ml of lysates) and incubated for 30 min at room temperature with gentle shaking. The mixture was poured into a column and washed with 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 20 mM imidazole. The protein was eluted using the same buffer containing 0.5 M imidazole. Elution samples were run on a 10% SDS Tris-glycine PAGE and analyzed by Western blotting with anti-RapA and anti-RapB antiserum.

**In vitro cross-linking of RapA was carried out in 50 mM HEPES (pH 7.0), 50 mM KCl, 20 mM MgCl2, RapA (10 μM) was incubated with and without EGS (5 mM) for 30 min at room temperature. After the addition of SDS loading buffer, the samples were analyzed on a 7% Tris–Tricine SDS–PAGE and stained with Coomassie Brilliant Blue.

**Immunological Methods**—A polyclonal antibody against RapA was obtained by standard procedures after immunization of rabbits with the purified protein (16). Antibodies against the PhrA peptide were obtained by immunizing the rabbits with a synthetic carboxyl-terminal 12-amino acid PhrA peptide (NH2-GKFKHAARNQT-COOH) conjugated at the amino terminus with keyhole limpet hemocyanin (17). The IgG fraction was purified by the caprylic acid-ammonium sulfate precipitation method (18, 19). Enrichment for PhrA-specific antibodies was obtained by removing the keyhole limpet hemocyanin contaminant immunoglobulin. Antigen-affinity purification was carried out using a keyhole limpet hemocyanin-agarose column (17). Unbound proteins were collected and concentrated with a Centriprep 10 (Amicon) to a final concentration of 1.7 mg/ml.

Western blotting was performed by standard techniques (20). After separation by SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). Proteins were visualized with specific antibodies and horseradish peroxidase anti-rabbit IgG conjugates using the ECL system from Amersham Biosciences.

**RESULTS**

**Oligomeric Status of RapA and RapB**—We previously reported the overexpression and purification of His-tagged RapA and RapB proteins active in dephosphorylating the phosphorylated form of the Spo0F response regulator (8). The activity of the proteins, however, was extremely labile. In the present studies, the same expression systems were used, but some modifications in the purification procedure were introduced that allowed us to obtain stable, Rap phosphatases.

Because RapA and RapB showed a significant level of aggregation when run on native gels and the amino acid sequence of RapA consists of 4 and 3 cysteine residues, respectively, we carried out all the purification steps in the presence of a reducing agent (20 mM β-mercaptoethanol or DTT 10 μM), which seemed to prevent protein aggregation. A further improvement to protein stability was provided by the addition of 20% glycerol. The reducing agent could be omitted without compromising the stability of the protein as long as this was constantly maintained in an argon-saturated environment.

The molecular masses of the His-tagged modified RapA and RapB proteins were calculated to be 48,090 and 47,557 Da,
Oligomeric status of RapA and RapB. A, purified RapA and RapB were analyzed by native gel electrophoresis on various concentrations of polyacrylamide. Represented is the 10% Tris-Tricine native gel containing RapA (lane 1), RapB (lane 2), BSA (lane 3), chicken egg albumin (lane 4), &lactoglobulin (lane 5), and carbonic anhydrase (lane 6). The shown molecular masses of the Rap proteins were calculated from the calibration curve generated as described in “Experimental Procedures.” B, in vitro cross-linking of purified RapA with EGS. RapA (10 μM) was incubated alone or in the presence of EGS (5 mM) before electrophoresis on a 10% Tris-Tricine-SDS gel. Lane 1, molecular mass markers, with values indicated in kDa. Lane 2, RapA alone; Lane 3, RapA incubated with EGS.

The size of the purified proteins, as observed on SDS-PAGE, was consistent with the predicted molecular masses (data not shown). To estimate the size of RapA and RapB in native conditions, we analyzed the purified proteins by electrophoresis on a set of Tris-Tricine native gels of various polyacrylamide concentrations (9, 10, 11, and 12%). The estimated molecular masses of RapA and RapB were 96 and 92 kDa, respectively (Fig. 1A). Using matrix-assisted laser desorption ionization time-of-flight mass spectrometry, the RapA protein was found to be in two species of 47,976 and 95,777 Da (data not shown).

An additional estimate of the molecular weights was obtained from in vivo and in vitro cross-linking studies carried out on RapA. Cross-linking of purified RapA with DST (not shown) or EGS (Fig. 1B) showed the presence of dimeric protein on a denaturing polyacrylamide gel. In vivo cross-linking experiments were carried out with the EGS reagent on cells of strain JHRAH carrying a rapA gene modified to encode a RapA protein with a 6× histidine tag at the carboxyl-terminal end. Immunodetection using a polyclonal antibody raised against RapA after electrophoresis in denaturing conditions revealed the presence of RapA in monomeric and dimeric form in addition to a higher (>100 kDa) form. At this time, we cannot discern between the possibility that this high molecular weight form is a multimer of RapA or a complex with other proteins (data not shown). These results suggest that the native state of RapA and RapB is most likely the dimer form.

Optimization of RapA Reaction Conditions—The reaction conditions for RapA-dependent dephosphorylation were investigated using purified phosphorylated Spo0F−P. RapA activity was first analyzed in the pH range 6.0–11 by carrying out the reaction in various buffers at room temperature. MES buffer was used for pH 6.0–6.5, HEPES buffer for pH 7.0–7.5, TAPS buffer for pH 8.0–9.0, CHES buffer for pH 9.0–10.0, and CAPS buffer for pH 10.0–11.0. Phosphatase activity had a sharp optimum at neutral pH. This requirement was confirmed when the Bis-Tris propane buffer was used in the pH range 6.5–9.5 (data not shown). The buffer HEPES at pH 7.0 was thus used for all subsequent experiments. The optimal salt concentration was then analyzed using KCl in the concentration range of 0–200 mM, and a sharp optimum was obtained at 50 mM (data not shown). Magnesium ions were required at 1 mM for optimal activity, but increasing concentrations did not significantly affect the rate of the reaction (data not shown). The requirement for divalent metal ions was further investigated on both RapA and RapB using CaCl2 and MnCl2 in addition to MgCl2 (Fig. 2). The fastest rate of dephosphorylation was obtained in the presence of manganese ions for both RapA- and RapB-dependent dephosphorylation as well as for Spo0F−P autodephosphorylation. In particular, the percentage of phosphate released from Spo0F−P was measured in an autodephosphorylation reaction (black bar), RapA-dependent dephosphorylation reaction (white bar), and RapB-dependent dephosphorylation reaction (gray bar). The reactions were carried out with 2.5 μM Spo0F−P and 1 μM RapA or RapB in reaction buffer alone (50 mM HEPES (pH 7.0), 10 mM DTT, 0.1 mM EDTA, 50 mM KCl, 20% glycerol) (lane 1, controls) or with the addition of 10 mM EDTA (lane 2), 5 mM CaCl2 (lane 3), 5 mM MgCl2 (lane 4), or MnCl2 (lane 5).

Interaction of RapA with Spo0F—RapA is a major antagonist of sporulation initiation by promoting the dephosphorylation of Spo0F−P. RapA does not dephosphorylate Spo0A−P or other phosphatases. In particular, the percentage of phosphate released by RapB in the presence of MnCl2 was 3- and 4-fold higher than that released in the presence of MgCl2 and CaCl2, respectively. The activity of RapA and RapB observed in the absence of metal ions is most likely due to carryover of some metal ions from the purification of the phosphatase proteins or Spo0F (Fig. 2, lane 1). This activity was in fact abolished in the presence of high levels of the metal chelator EDTA (10 mM) (Fig. 2, lane 2).
Biochemical Characterization of RapA

Fig. 3. Formation of the RapA-Spo0F–P complex. A, the 10% Tris-Tricine-EDTA native gel assay was used to detect the formation of the RapA-Spo0F–P complex. The indicated amounts of RapA (lanes 1–5) were added to a constant amount of Spo0F–P. Spo0F–P alone is shown in lane 1, whereas RapA alone is shown in lane 6. A reaction containing equimolar concentrations of the two proteins was allowed to proceed for 0, 15, and 30 min before loading on the 10% Tris-Tricine native gel (lanes 7–9) to demonstrate complex dissociation upon Spo0F dephosphorylation. B, Coomassie-stained 10% Tris-Tricine-EDTA native gel of RapA and unphosphorylated Spo0F. Lane 1, Spo0F (10 μM); lane 2, Spo0F (10 μM) and RapA (5 μM); lane 3, Spo0F (10 μM) and RapA (10 μM); lane 4, Spo0F (10 μM) and RapA (20 μM); lane 5, Spo0F–P (10 μM) and RapA (10 μM); lane 6, RapA (10 μM). C, Western blot analysis of the gel in C using Spo0F antibodies. Lanes are as in C.

response regulators tested (8). In this study, we investigated whether RapA-specific interaction was also limited to the phosphorylated form of Spo0F or would extend to the unphosphorylated protein as well.

We studied the interaction of RapA with Spo0F using a native gel electrophoresis assay. As shown in Fig. 3A, RapA and the Spo0F/Spo0F–P alone display different mobilities in a 10% Tris-Tricine-EDTA native gel. However, when mixed with each other before loading on the gel, a clear shift in the mobility of RapA was observed, indicating the formation of a complex. A titration of Spo0F–P with RapA showed that at a 1:1 molar ratio, RapA was fully sequestered in the complex. At a 1:2 ratio Spo0F–P/RapA, ~50% of RapA ran in a band with the same mobility of the protein alone (Fig. 3A, lanes 2–5). No band shift was observed when unphosphorylated Spo0F was used in the reaction (Fig. 3B, lanes 2–4). However, probing the gel with a Spo0F antibody indicated the presence of Spo0F protein at the level of the RapA protein (Fig. 3C, lanes 2–4). This suggests that RapA may interact with unphosphorylated Spo0F, but a stable complex with altered mobility is formed only with the phosphorylated form of Spo0F. This complex dissociated if the dephosphorylation reaction was allowed to proceed after complex formation (Fig. 3A, lanes 7–9), thus regenerating protein bands with the same mobility of RapA and Spo0F.

To investigate the composition of the presumed complex, the shifted band was excised from the native gel and analyzed by SDS-polyacrylamide electrophoresis as described under “Experimental Procedures.” The results indicated the presence of RapA and Spo0F at 1:1 molar ratio (data not shown).

The molecular mass of the RapA/Spo0F–P complex was determined by native polyacrylamide gel electrophoresis and compared with Spo0F and RapA alone. The complex was determined to be 121 kDa, a value significantly close to the sum of the molecular mass of 2 molecules of RapA and 2 molecules of Spo0F, calculated to be 126.6 kDa (data not shown). This suggested that the RapA-Spo0F–P complex consists of one RapA dimer and two Spo0F–P molecules.

Interaction of RapA with PhrA—The native gel electrophoresis assay used to study the RapA-Spo0F–P interaction was also applied to the parallel analysis of the interaction between RapA and its pentapeptide inhibitor PhrA (NH₂-ARNQT-COOH). We observed that at equimolar concentrations, the addition of pentapeptide resulted in a change of RapA mobility (Fig. 4A, lane 3). A 5:1 ratio RapA:PhrA did not affect the mobility of the phosphatase, whereas a 1:5 ratio did not increase the shift of the protein in the gel from that observed at equimolar concentrations. The mobility of RapA was not affected when an equimolar concentration of the PhrC pentapeptide (NH₂-ERGMT-COOH) was mixed with the phosphatase (data not shown).

To prove that the shifted band actually contained the PhrA pentapeptide complexed with RapA, the 0 arm length EDC cross-linker was added to the reactions and analyzed by denaturing (Fig. 4B) and native (not shown) polyacrylamide gel electrophoresis followed by Western blotting and immunodetection using polyclonal antibodies against RapA or PhrA. Detection of PhrA in the protein band corresponding to RapA (Fig. 4B, lane 10) in both electrophoresis conditions confirmed the formation of a complex between the two proteins. The increased mobility imparted to RapA by the presence of PhrA in native condition could be the result of a change in electrostatic charge or the induction of a conformational change in RapA upon complex formation.

PhrA Dissociates the RapA-Spo0F–P Complex—We utilized the native gel electrophoresis assay to analyze the interaction of RapA with PhrA and Spo0F–P concurrently. As shown in Fig. 4A (lanes 5–8), the addition of PhrA dissociated Spo0F–P from the complex with RapA. To quantify the level of dissociation, the experiment was carried out using ³²P-labeled Spo0F–P (Fig. 5). With the addition of PhrA at a molar ratio of 1:1:1, an equilibrium was reached with ~70% of RapA associated with PhrA and 30% associated with Spo0F–P (Fig. 5, A

---

2 S. Ishikawa, L. Core, and M. Perego, unpublished data.
FIG. 4. Formation of the RapA–PhrA complex prevents formation of the RapA–Spo0F–P complex. A, RapA (10 μM) (lanes 1–8) was mixed with increasing concentrations of PhrA and immediately run on a 10% Tris-Tricine-EDTA native gel. PhrA was added at 0, 2, 10, and 50 μM (lanes 1–4 and 5–8). Spo0F–P was added at 10 μM (lanes 5–9). B, An EDC-cross-linked RapA–PhrA complex was analyzed on a 10% Tris-Tricine SDS gel by Coomassie staining (lanes 1–4) and Western blotting using antibody against RapA (lanes 5–7) or against PhrA (lanes 8–10). Lane 1, molecular weight markers expressed in kDa; lanes 2, 5, and 8, RapA without cross-linker; lanes 3, 6, and 9, RapA cross-linked with EDC; lanes 4, 7, and 10, RapA mixed with PhrA and cross-linked with EDC. C, PhrA does not change the mobility of RapA892 and does not dissociate it from the complex with Spo0F–P. The native gel electrophoresis assay was carried out with the RapA892 mutant protein (10 μM) (lanes 1–4) or RapA892 and Spo0F–P (10 μM each) (lanes 7–10). The PhrA pentapeptide was added at the indicated concentrations immediately before loading the gel. As a reference, the wild-type RapA protein (10 μM) (lane 5) and the RapA–PhrA (10 μM each) complex (lane 6) were loaded on the same gel.

and B, lanes 6). The same equilibrium was reached when the phosphorylated form of Spo0F was added to the preformed RapA–PhrA complex (Fig. 5, A and B, lanes 5). Thus, the pentapeptide inhibitor can displace Spo0F–P after the complex of the substrate with the enzyme is formed and vice versa, Spo0F–P can partially displace the pentapeptide. The equilibrium reached suggests that substrate and peptide might have a different affinity for RapA.

PhrA Does Not Dissociate the RapA892–Spo0F–P Complex—We previously showed that a strain carrying the rapA892 mutation was sporulation-deficient due to the inability of the RapA892 protein to respond to the PhrA pentapeptide inhibitor (10). This resulted in a protein constitutively active in dephosphorylating Spo0F–P, thus inhibiting the initiation of the sporulation pathway. Here we used the native gel electrophoresis assay to determine whether the lack of sensitivity to PhrA was due to RapA892 inability to bind the pentapeptide.

The RapA892 protein was purified after the protocol established for the wild-type protein, mixed with various concentrations of PhrA pentapeptide, and analyzed by the native gel assay. As shown in Fig. 4C, the mutant protein formed a stable complex with Spo0F–P. However, its electrophoresis mobility did not change upon the addition of PhrA, and the RapA892–Spo0F–P complex was not displaced even in the presence of a 5-fold excess of peptide versus Spo0F–P. This could suggest that RapA892 is indeed unable to form a complex with the pentapeptide. However, probing of EDC-cross-linked RapA892 and PhrA with the corresponding antibodies showed the presence of pentapeptide in the protein band with the mobility of the phosphatase (data not shown). These results suggest that even though the pentapeptide may interact with the RapA892 protein transiently, it is unable to form a stable complex that can displace the Spo0F–P.

FIG. 5. Equilibrium of RapA association with PhrA and Spo0F–P. The effect of PhrA on the RapA–Spo0F–P complex and the effect of Spo0F–P on the RapA–PhrA complex was analyzed on a 10% Tris-Tricine-EDTA native gel. Proteins and peptides at 10 μM each were mixed at room temperature and immediately stopped by the addition of loading dye and incubation in ice prior to loading on the gel. A, Coomassie-stained gel. Lane 1, RapA; lane 2, RapA + PhrA; lane 3, Spo0F–P; lane 4, complex RapA + Spo0F–P; lane 5, PhrA was added to the pre-formed RapA–Spo0F–P complex; lane 6, PhrA–P was added to the preformed RapA–PhrA complex. B, Autoradiograph of the gel shown in A. Lanes are as in A.

DISCUSSION

This study shows that the RapA and RapB phosphatases are dimeric proteins with an optimal activity at pH 7 in the presence of 50 mM salt. The phosphatase activity is dependent upon the presence of at least 1 mM divalent cations, with manganese supporting the highest level of activity.

We observed that the ability of RapA and RapB to dephosphorylate Spo0F–P depends on maintaining a strong reducing environment during protein purification. If oxidized, RapA and, to a lesser extent, RapB form inter- and/or intramolecular disulfide bonds and aggregate, thus losing their dephosphorylation activity. The predicted amino acid sequence of RapA and RapB indicate the presence of four and three cysteine residues, respectively (8). It is believed that disulfide bond formation is prevented in cytoplasmic proteins due to the reducing environment of the cytoplasm (21, 22). This is consistent with our observation that Rap stability in vitro requires strong reducing conditions or an argon-saturated environment. Transiently formed disulfide bonds are known to be part, for example, of the
catalytic cycle of enzymes such as ribonucleotide reductase or in redox-regulated proteins such as OxyR (23, 24). Preliminary mutagenesis studies indicate that none of the RapA cysteine residues are necessary for protein activity in vitro, thus excluding their involvement in a functional or structural role. Nevertheless, whether the cysteine residues in Rap phosphatases may be part of an additional negative regulatory mechanism remains to be defined.

A major finding of this study is that the RapA dimer forms a stable complex with two molecules of phosphorylated Spo0F. Although interaction with the unphosphorylated response regulator is detected, the presence of the phosphoryl group is required to obtain a complex that is stable during electrophoresis. Dephosphorylation of Spo0F–P after complex formation results in dissociation of the two proteins. Dissociation of the RapA-Spo0F–P complex is also induced by the PhrA pentapeptide inhibitor. In the present study we observed that at equimolar concentrations, an equilibrium is reached in which ~70% of RapA is complexed with PhrA, whereas 30% is complexed with Spo0F–P. Whether this observation reflects a different level of affinity for the Rap phosphatase by its substrate or its inhibitor remains to be established by more detailed analysis.

Rap phosphatases are composed of a tandem series of repeated motifs related to the tetratricopeptide repeat (TPR) (25). Six TPR motifs have been identified in the members of the Rap family. The TPR motif has been found in a wide variety of proteins from prokaryotes to eukaryotes, and it is generally believed to represent an ancient protein-protein interaction module (26). Each motif is composed of 34 amino acids and folds into a well defined structure that consists of a pair of antiparallel α helices of equivalent length generally associated with a packing angle of ~24° between the helix axes (27). TPR motifs are generally organized in tandem arrays of 3–16 motifs that form a scaffold with a right-handed superhelical structure featuring a helical groove suitable for recognizing target proteins (28). Recently, the structure of the amino- and carboxyl-terminal TPR domains of the Hop protein complexed with the carboxyl-terminal peptides of the Hsp70 and Hsp90 chaperones have been determined by x-ray crystallography (29). These structures show the peptide in an extended conformation spanning the groove in the TPR domains. Modeling of the six TPR motifs of RapA showed that a helical groove with a predominantly negative charge will be formed. We have proposed that this could provide the appropriate binding environment for the interacting partners PhrA and Spo0F (25). The arginine residue at position 2 of the pentapeptide (ARNQT) is a key residue in Phr peptides known to be active (10). Also, the positively charged Arg-16 on helix α1 and Lys-56 on loop β3-a3 could be interacting with the groove, as they have been found to be involved in Spo0F interaction with RapB (30).

Biochemical and structural studies are in progress that will allow us to identify the sites of interaction between RapA and its substrate and its inhibitor. Mutagenesis studies indicated that Spo0F residues involved in interaction with Rap phosphatases are localized on the top of the molecule near the active site, mainly in the β1-α1, β3-α3, and β4-α4 loops connecting the β strands to the α helices of the response regulator (30). The same residues were also found to be involved in Spo0F interaction with histidine kinases and the Spo0B phosphotransferase (12) as stated above.

Also to be determined is whether PhrA and Spo0F–P interact with RapA in a competitive manner or not. The structural organization of RapA in TPR motifs allows us to envision two equally plausible scenarios, one with the pentapeptide binding within the RapA groove in as much as the Hsp peptides bind to the Hop protein, perhaps inducing a conformational change that prevents Spo0F–P from binding to RapA at a different site (29). Note that a conformational change of the TPR domains of the Hop protein upon peptide binding are not expressively reported, but it is predicted that the TPR helix formed by TPR-containing proteins may allow such proteins to interact simultaneously with multiple target proteins by utilizing specific combinations of TPR motifs within the superhelical structure. Alternatively, PhrA and Spo0F may compete for the same binding site, likely to be in the RapA groove (Fig. 6).

We previously showed that inhibition of RapA by PhrA is highly specific and dependent on the amino acid sequence of the pentapeptide. We proposed that specificity was probably the result of critical contacts of the peptide side chains with RapA residues (10). Particularly important is also the interaction of the carboxyl-terminal carboxylate group as its substitu-

FIG. 6. Model of RapA interaction with the substrate Spo0F–P and the PhrA pentapeptide inhibitor. RapA is schematically represented on the basis of a predicted three-dimensional structure modeled on available coordinates of TPR-containing proteins (25). A mechanism of competitive inhibition is envisioned, but alternative mechanisms cannot be excluded.

3 L. Core and M. Perego, unpublished data.

4 Stephenson, S., and Perego, M. (2002) Mol. Microbiol. in press.
tion with a carboxyl-terminal amide reduces approximately 40-fold the activity of the peptide (11). The interaction established by the carboxyl-terminal carboxylate, however, may not necessarily be involved in specificity because it is a common feature of Phr peptide activity, but rather, may only have a role in positioning the peptide with respect to the TPR domain. The structural alignment of two TPR domains of the Hop protein complexed with their respective Hsp70 and Hsp90 carboxyl-terminal peptides suggests in fact that, whereas the last two residues of the peptides are bound at equivalent positions in the respective TPR domains, the remaining amino-terminal parts of the peptides are binding to very different TPR regions, thus establishing the specificity of contact (29). If a similar mechanism of recognition and specificity were used in the interaction of Phr peptide with Rap phosphatases, we can imagine PhrA making contact first and then interacting specifically with RapA so that a stable complex is formed. This complex is visualized by the gel electrophoresis assay developed in this study. Thus specificity will be established only between paired proteins (RapA-PhrA or RapE-PhrE for example), but contacts could be made across pairs. That contacts occur without the establishment of stable interactions is suggested by the observation that the PhrA peptide can be found cross-linked to RapB, although it does not affect its phosphatase activity.

Acknowledgment—We acknowledge Darshini Mehta for the purification of the PhrA antibody.

REFERENCES

1. Hoch, J. A. (2000) Cur. Opin. Microbiol. 3, 165–170
2. Jiang, M., Shao, W., Perego, M., and Hoch, J. A. (2000) Mol. Microbiol. 38, 535–542
3. Burbulyte, D., Trach, K. A., and Hoch, J. A. (1993) Cell 64, 545–552
4. Trach, K. A., Burbulyte, D., Spiegelman, G., Perego, M., Strauch, M. A., Day, J., and Hoch, J. A. (1999) in Genetics and Bio/Technology of Bacilli. (Zukowski, M. M., Ganesan, A. T., and Hoch, J. A., eds) Vol. 3, pp. 357–365, Academic Press, Inc., San Diego, CA
5. Perego, M. (1998) Trends Microbiol. 6, 366–370
6. Jiang, M., Grau, R., and Perego, M. (2000) J. Bacteriol. 182, 303–310
7. Kunst, F. et al. (1997) Nature 390, 249–256
8. Perego, M., Hanstein, C. G., Welsh, K. M., Djavakhishvili, T., Glauser, P., and Hoch, J. A. (1994) Cell 79, 1047–1055
9. Mueller, J. P., Bukusoglu, G., and Sonenshein, A. L. (1992) J. Bacteriol. 174, 4361–4373
10. Perego, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8612–8617
11. Cori, L. J., Ishikawa, S., and Perego, M. (2001) Peptides 22, 1549–1553
12. Tseng, Y.-L., and Hoch, J. A. (1997) J. Mol. Biol. 272, 260–212
13. Perego, M. (1993) in Bacillus subtilis and Other Gram-Positive Bacteria: Biochemistry, Physiology, and Molecular Genetics (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds) pp. 615–624, American Society for Microbiology, Washington, D. C.
14. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
15. Scheaffer, P., Millet, J., and Aubert, J. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 701–711
16. Hanly, W. C., Artwohl, J. E., and Bennett, B. T. (1995) Inst. Lab. Anim. Res. J 37, 93–118
17. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Steinbuch, M., and Audran, R. (1969) Arch. Biochem. Biophys. 134, 279–284
19. Russ, C., Callegaro, I., Lanza, B., and Ferrone, S. (1983) J. Immunol. Methods 65, 269–271
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1982) Molecular Cloning: A Laboratory Manual, pp. 18–1.88, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Gilbert, H. F. (1996) Adv. Enzymol. Relat. Areas Mol. Biol. 63, 69–172
22. Hwang, C., Sinakev, A. J., and Lodish, H. F. (1992) Science 257, 1496–1502
23. Zheng, M., Aslund, F., and Storz, G. (1998) Science 279, 1718–1721
24. Abberg, A., Hahne, S., Karlsson, M., Larsson, A., Orn, M., Ahgren, A., and Sjoberg, B. M. (1989) J. Biol. Chem. 264, 12249–12252
25. Perego, M., and Brannigan, J. A. (2001) Peptides 22, 1541–1547
26. Blatch, G. L., and Lassele, M. (1999) Bioessays 21, 932–939
27. Das, A. K., Cohen, P. T. W., and Barford, D. (1998) EMBO J. 17, 1192–1199
28. Groves, M. R., and Barford, D. (1999) Curr. Opin. Struct. Biol. 9, 383–389
29. Scheufler, C., Brinker, A., Borek, G., Pegoraro, S., Moroder, L., Bartunik, H., Hartl, F. U., and Moarefi, I. (2000) Cell 101, 199–210
30. Tseng, Y.-L., Feher, V. A., Carvagly, J., Perego, M., and Hoch, J. A. (1998) Biochemistry 37, 16538–16545