Purification and Characterization of Bacillus subtilis PyrR, a Bifunctional pyr mRNA-binding Attenuation Protein/Uracil Phosphoribosyltransferase*

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Bacillus subtilis PyrR has been shown to mediate transcriptional attenuation at three separate sites within the pyrimidine nucleotide biosynthetic (pyr) operon. Molecular genetic evidence suggests that regulation is achieved by PyrR binding to pyr mRNA. PyrR is also a uracil phosphoribosyltransferase (UPRTase). Recombinant PyrR was expressed in Escherichia coli, purified to homogeneity, physically and chemically characterized, and examined with respect to both of these activities. Mass spectroscopic characterization of PyrR demonstrated a monomeric mass of 20,263 Da. Gel filtration chromatography showed the native mass of PyrR to be dependent on protein concentration and suggested a rapid equilibrium between dimeric and hexameric forms. The UPRTase activity of PyrR has a pH optimum of 8.2. The $K_m$ value for uracil is very pH-dependent; the $K_m$ for uracil at pH 7.7 is $990 \pm 114$ μM, which is much higher than for most UPRTases and may account for the low physiological activity of PyrR as a UPRTase. Using an electrophoretic mobility shift assay, PyrR was shown to bind pyr RNA that includes sequences from its predicted binding site in the second attenuator region. Binding of PyrR to pyr RNA was specific and UMP-dependent with apparent $K_m$ values of 10 and 220 nM in the presence and absence of UMP, respectively. The concentration of UMP required for half-maximal stimulation of binding of PyrR to RNA was 6 μM. The results support a model for the regulation of pyr transcription whereby termination is governed by the UMP-dependent binding of PyrR to pyr RNA and provide purified and characterized PyrR for detailed biochemical studies of RNA binding and transcriptional attenuation.

The Bacillus subtilis pyrimidine biosynthetic (pyr) operon encodes all of the enzymes for the de novo biosynthesis of UMP and two additional cistrons encoding a uracil permease and the regulatory protein PyrR (1–4). On the basis of molecular genetic evidence it was proposed that PyrR regulates transcription whereby termination is achieved by PyrR binding to pyr mRNA. PyrR is proposed to regulate the ratio of terminated to readthrough transcripts at each attenuation site by permitting the formation of a α-independent transcription terminator when exogenous pyrimidines are available. The binding of PyrR to pyr mRNA interferes with the formation of an alternative upstream stem-loop structure, the antiterminator, which is otherwise kinetically and thermodynamically favored. The presence of a conserved sequence within the 5'-stem of each antiterminator suggested a site within the pyr mRNA for interaction with PyrR (3); this site is the locus of several cis-acting mutants in the first pyr attenuator which are deficient in repression by pyrimidines (6).

In addition, PyrR functions as a novel uracil phosphoribosyltransferase (UPRTase),1 catalyzing the formation of UMP and pyrophosphate from uracil and 5-phosphoribosyl α-1-pyrophosphate (PRPP) despite its lack of primary sequence similarity to other known UPRTases (3, 7). The UPRTase activity of PyrR was first discovered by Ghim and Neuhard (8), who characterized the pyrR gene from Bacillus caldolyticus. The role of the enzymatic function of PyrR is not known; Bacillus subtilis possesses an additional UPRTase that has been shown to be quantitatively more important than PyrR (3, 7). It has been demonstrated that UMP and PRPP function as negative and positive regulators, respectively, of the pyr operon (3, 5, 9).

Direct biochemical characterization of this attenuation mechanism is required to test the above proposals. In this paper we describe the purification of PyrR. Physical and enzymatic properties of PyrR are described, and the ability of PyrR to bind specifically to pyr mRNA is demonstrated.

EXPERIMENTAL PROCEDURES
Bacterial Strains, Plasmids, Media, and Growth
Escherichia coli strains DH5α (Life Technologies, Inc.) and TG1 (10) were used for plasmid construction and plasmid propagation for purification. Luria broth (11) was used for the growth of cultures for plasmid expression and purification of PyrR. Bacterial strains used for plasmid construction and plasmid propagation for purification were deficient in repression by pyrimidines (6).

Expression and Purification of PyrR
B. subtilis PyrR was expressed in E. coli from plasmid pTSROX3 (pUC18 (12) into which a 0.79-kilobase pair EcoRI-SphI fragment bearing the pyr promoter, 5'-leader, and pyrR from pTS185 (3) was inserted), in which PyrR expression was driven by tandem lac and pyr promoters. E. coli SØ408 (relA1 rpsL254 metB1 upp-11, from J. Neuhard, University of Copenhagen), a strain lacking endogenous UPRTase activity, bearing pTSROX3 was grown in 3 liters of Luria broth (11) to an optical density of 1.0. E. coli were harvested by centrifugation at 4 °C.
broth, supplemented with 97 µg of ticarcillin and 3 µg of clavulanic acid/ml of culture. The cells were harvested by centrifugation, washed in cold 0.9% NaCl, and stored at −80 °C.

All enzyme purification procedures were performed at 4 °C. The pH values for Tris buffers were determined at 25 °C. The cells were resuspended with 8 ml of 100 mM Tris acetate, pH 7.0, per g of cell paste, disrupted by sonication on ice, and cell debris removed by centrifugation.

Streptomycin sulfate (0.11 volume of a 10% solution freshly prepared in 100 mM Tris acetate, pH 7.0) was added, and the precipitate was removed by centrifugation. Further contaminants were precipitated with 0.558 volume of saturated ammonium sulfate, which was resuspended with 100 mM Tris acetate, pH 7.0, and removed by centrifugation. PyrR was precipitated from the supernatant solution by the addition of 0.857 volume of buffered saturated ammonium sulfate and collected by centrifugation. The precipitate was resuspended in 4 ml of buffer R (100 mM Tris acetate, pH 7.0, 10 mM Na2P04, 100 mM NaCl) for every g of the cell paste used. The solution was dialyzed against buffer R.

The entire sample was loaded onto a 2.2 × 21-cm Q-Sepharose Fast Flow (Pharmacia) column that had been equilibrated in buffer R. Buffer R (400 ml) was allowed to flow through the column, and PyrR was eluted using an 800-ml linear NaCl gradient, from 0 to 100 mM, in 100 mM Tris acetate, pH 7.0, 10 mM Na2P04. Fractions from the trailing half of the PyrR peak, which eluted at a conductivity equivalent to 2193 ppm NaCl, were pooled. The purified PyrR sample was then concentrated approximately 6-fold by pressure dialysis.

Additional impurities were precipitated with an equal volume of saturated ammonium sulfate and removed by centrifugation. PyrR was precipitated from the supernatant fluid by the addition of another equal volume of saturated ammonium sulfate, then collected by centrifugation. The precipitate was dissolved in 0.5 ml of 10 mM Tris acetate, pH 7.5, per g of cell paste used and dialyzed against 10 mM Tris acetate, pH 7.5. The dialyzed solution was divided into aliquots and stored at −80 °C.

UPRTase Assay

UPRTase activity was determined by measuring the conversion of [2-14C]uracil to [14C]UMP by modifications of the method of Rasmussen et al. (13).

Method 1—Method 1 was used during development of the purification of PyrR. The assay conditions were pH 9.0 at 37 °C, 50 mM Tris acetate, 20 mM 2-mercaptoethanol, 5 mM MgCl2, 1.2 mM PRPP, and 0.1 mM [14C]uracil (about 4.1 × 104 dpm/assay). The assay mix (20 µl, containing all components but PRPP) was combined with 20 µl of enzyme, which was diluted in 0.1 M Tris acetate, pH 9.7, and 1 mg/ml BSA. Reactions were preheated for 1 min at 37 °C, initiated with 10 µl of 6 mM PRPP, and incubated for 5 min at 37 °C. (For purified PyrR after the Q-Sepharose step it was necessary to stabilize the enzyme by including 3 mM PRPP in the assay mix and initiating the reactions with 10 µl of 0.5 mM [14C]uracil.) The reactions were stopped by heating at 100 °C for 1 min. Separation of the product [14C]UMP from [14C]uracil on DEAE-cellulose paper was essentially as described for Method 2 below.

Method 2—After purified PyrR was available the assay was further optimized, and Method 2 was used for kinetic characterization of the purified protein. Each assay contained 20 µl of 0.125 M buffer, 12.5 mM MgCl2, and 2.5 times the desired final concentration of PRPP. PyrR was diluted into 100 µl buffer containing 1 mg/ml BSA immediately before assay, and 20 µl was mixed with 20 µl of assay mix and incubated at 37 °C for 5 min. Reactions were initiated by the addition of 10 µl of [14C]uracil at 5 × the desired final concentration (1.5–6 × 105 cpm). Reactions occurred at 37 °C, and 5-µl samples taken at various reaction times were spotted onto 1-cm2 squares of DEAE-cellulose paper (Whatman) and dried rapidly. The spotting was found to stop the reaction very quickly, so heating was not necessary. The DEAE-cellulose paper was washed four times for 20 min each with water and once for 15 min with methanol. To determine the total 14C counts in each reaction mixture duplicate 5-µl samples from each tube were spotted onto squares of DEAE-cellulose which were not washed. Radioactivity on the DEAE-cellulose paper squares was determined by liquid scintillation. Kinetic data were analyzed using a program written on a computer (IntelliKinetics, State College, PA) computer program. At least five concentrations of each substrate were used at each pH value reported; the data fit well to a Ping Pong Bi Bi rate equation at all pH values.

Electrospray Mass Spectroscopic Analysis of PyrR

For ESI-MS, PyrR was dissolved in 50% acetonitrile (v/v) and 0.1% formic acid (v/v) solution by repeated desalting using a Microcon™ 10 concentrator (Amicon, Beverly, MA) until all salts were reduced to picomolar concentrations. The sample was submitted to the University of Illinois School of Chemical Sciences Mass Spectrometry Laboratory for ESI-MS analysis on a VG Quattro (quadrupole-hexapole-quadrupole) mass spectrometer system (Fisons Instruments, VG Analytical, Manchester, U. K.). Data acquisition and processing were controlled by the VG MassLynx (version 2.0) data system (Micromass, Manchester, U. K.). MaxEnt (maximum entropy) software (Micromass) was used for the processing and analysis of zero charge state electrospray data.

Sulphydryl Group Titration

Cysteinyl sulphydryl groups were titrated with 4 mM 5,5'-dithio-bis(2-nitrobenzoate), measuring the increase in absorbance at 412 nm as described by Ellman (14). The buffer used for PyrR was 0.1 x 1 K, pH 7.3, containing 1 mM EDTA; for denatured PyrR the buffer was 0.1 x Tris-Cl, 1 mM EDTA, 0.5% SDS, pH 8.4. The concentration of PyrR was determined from its extinction coefficient of 7,100 m−1 cm−1 at 275 nm.

Gel Filtration Analysis of PyrR

A column (1-cm diameter, 95-cm height, 75-ml bed volume) of Sephadex G-150 (Pharmacia Biotech Inc.) was used to determine the native molecular weight of PyrR. The buffer used was 100 mM Tris acetate, pH 7.5. The column was loaded with 0.6–1.0-ml samples of PyrR and eluted at 4 °C. Proteins used to control a M, standard curve for the column were myoglobin, chicken serum albumin, yeast hexokinase, and bovine γ-globulin. The protein concentrations in the eluted fractions determined by their absorbance at 280 nm; when the protein was too dilute or the fractions containedUMP, the protein was determined using the Bradford method (15) with reagents purchased from Bio-Rad.

Preparation of Transcription Templates

A template for in vitro run-off transcription of pyr mRNA nucleotides +682 to +761, which correspond to the anti-antiterminator (or “binding loop”) from the pyrR-pyrP intercistronic region, was created using PCR. The PCR was performed using the forward primer 5'- CGGAATTC-ATGAATCTACATTAGGAGATATGAAAGCGAATTAATGCACCT- CTTTATA-3' (where an BamHI site is underlined), and the pLS361 plasmid (5) as template. The purified PCR product was digested with EcoRI and BamHI and ligated into similarly digested pUC18 to create pBSBL2. This plasmid template was linearized using MluI before its inclusion in the transcription reaction.

Similarly, templates for in vitro run-off transcription of pyr mRNA nucleotides +722 to +796, +772 to +809, which correspond to the antiterminator and terminator, respectively, from the pyrR-pyrP intercistronic region, were created using PCR. The antiterminator template pBSAT2 was synthesized using the primers 5'- CGGAATTCCTACTA- TCTACAATATAGGAGATATGAAAGCGAATTAATGCACCT- CTTTATA-3' (where an EcoRI site is underlined and is immediately upstream of a bacteriophage T7 promoter in italics), the reverse primer 5'- CGGGATCTCTTTTTTGCGAGTGTGTTGTTGGT-3' (where a BamHI site is underlined), and the pLS361 plasmid (5) as template. The purified PCR product was digested with EcoRI and BamHI and ligated into similarly digested pUC18 to create pBSAT2. This plasmid template was linearized using MluI before its inclusion in the transcription reaction.

The terminator template pBST2 was synthesized using the primers 5'- CGGAATTCACCAGCGGTATTCATTTACTAAGTGACATCAGAA- AAGCGGAGGATACAAAG-3' (where a BamHI site is underlined, and an MluI site is in italics), and pLS361 as template. The purified PCR product was digested with EcoRI and BamHI and ligated into similarly digested pUC18 to create pBST2. This plasmid template was linearized with XbaI before its inclusion in the transcription reaction. The terminator template pBST2 was synthesized using the primers 5'- CGGAATTCCTACTA- TCTACAATATAGGAGATATGAAAGCGAATTAATGCACCT- CTTTATA-3' (where an EcoRI site is underlined and is immediately upstream of a bacteriophage T7 promoter in italics), 5'- CGTGCTAGACCT- CTTGCTTTTTTACGGAGGTTAAGCGGACGTAATTT-3' (where an XbaI site is underlined), and pLS361 as template. The purified PCR product was digested with EcoRI and XbaI and ligated into similarly digested pUC18 to create pBST2. This plasmid template was linearized with XhoI before its inclusion in the transcription reaction.

To create a template that contained the same pyr nucleotides as pBSBL2 but was designed to transcribe the antisense RNA strand corresponding to the anti-antiterminator, two synthetic DNA oligonucleotides were created using the template to produce run-off transcripts directly. The first oligonucleotide, or “top strand,” corresponds to the core T7 promoter sequence (5'- TATAATCACGATC-3'), and the second oligonucleotide, or “bottom strand,” has a sequence that anneals to the T7 promoter at one end and also nucleotides that are complementary to the desired pyr transcript (pyr mRNA nucleotides +761 to +862). The sequence of the bottom strand oligonucleotide was 5'- TATGGAGAAGGATATGACATCCTTTTATAGGGC-
Preparation of pyr RNA

pyr RNA for use in the electrophoretic gel mobility shift assay was prepared by in vitro run-off transcription using the MEGAScript™ kit from Ambion (Austin, TX) as described by the manufacturer, except that 1 µl of 75 mM ATP diluted 1:500 in RNase-free H₂O and 5 µl of [α-³²P]ATP (3,000 Ci/mmol, 10 mCi/ml, ICN, Costa Mesa, CA) were added to each 20 µl reaction mixture. The full-length RNA product was purified by electrophoresis on a denaturing 20% polyacrylamide gel (19:1, acrylamide:bisacrylamide) containing 8 M urea. The RNA was visualized by autoradiography, excised, and eluted with 1 ml of 0.5 M ammonium acetate, 10 mM MgSO₄, 0.1% SDS, 1 mM EDTA in RNase-free H₂O at room temperature for at least 3 h. The elution solution was extracted twice with acid phenol:chloroform and precipitated with ethanol.

Preparation of Nonspecific RNAs

Nonspecific RNA was prepared as above but using the plasmid templates as follows. An 18 S rRNA transcript was prepared using the template provided with the Ambion MEGAScript™ kit; an actin transcript was prepared using the template provided with the Ambion MAXIscript™ kit; and an RNA corresponding to multiple cloning site DNA was prepared using pSP72 (Promega, Madison, WI) linearized with HindIII.

Electrophoretic Mobility Shift Assay for RNA Binding

Gel shifts were performed using a Bio-Rad PROTEAN® Ixi electrophoresis apparatus with the core cooled to 2 °C and the buffer recirculating between the upper and lower reservoirs. All gel shifts were run on 6% native polyacrylamide (79:1, acrylamide:bisacrylamide) gels containing 12.5 mM Tris acetate, pH 7.5, and 2.5% glycerol (v/v), using 12.5 mM Tris acetate, pH 7.5, containing 1 mM magnesium acetate as running buffer. Gels were pre-run at 150 V for 90 min and then cooled by recirculation for 1 h. RNA-binding reaction mixtures were loaded onto the gel with tracking dyes in a separate lane. The gel was subjected to electrophoresis at 30 V for 15 min followed by 300 V for 5 h. The gel was blotted onto filter paper, dried, and radioactivity was visualized by exposing the gels to x-ray film. For quantitation of the binding data, the dried gel was exposed to a storage PhosphorImage screen (Molecular Dynamics, Sunnyvale, CA), after which the data were quantitated with a PhosphorImager using the ImageQuant software. Binding curves were fit to hyperbolae, and binding constants were calculated using the KinetAsyst computer program as described above.

RNA-binding reaction mixtures were assembled on ice. The binding conditions were modified from the procedure of Batey and Williamson (17). Each reaction contained 16 µl of binding mix, 2 µl of PyrR (diluted as described below), and 2 µl RNA (prepared as described below; final concentration of 50 pmol RNA). The binding mix gave final assay concentrations of 10 mM HEPES-KOH (pH 7.5), 50 mM potassium acetate, UMP (when added), 1 mM magnesium acetate, 0.1 mM EDTA, 0.1 mg/ml yeast tRNA, 5 µg/ml bactin, 0.01% Igepal CA-630 (Sigma; Igepal CA-630 is an analog of Nonidet P-40), and 0.08 unit/ml placental RNase inhibitor (Ambion). PyrR was diluted using 12.5 mM Tris acetate buffer, pH 7.5, which contained 1 mg/ml RNase-free acetylated BSA (U. S. Biochemical Corp.). Because PyrR has poor thermostability at high dilution, the protein must be thawed on ice and diluted immediately before use. To minimize alternate RNA secondary structures, the RNA was heated to 75 °C for 15 min, slow cooled for 1 h, and cooled on ice for 5 min before its incorporation into the binding mixture. The completed binding reactions were incubated on ice for 1 h before loading on the gel. Immediately before loading them onto the gel, 2 µl of RNase-free 50% glycerol was added to the reaction mixtures.

RESULTS AND DISCUSSION

Overexpression and Purification of PyrR—B. subtilis DNA specifying the pyr promoter, 5′-leader, and pyrR was cloned into pUC18 to generate plasmid pTSROX3, in which the expression of pyrR was driven from the tandem lac and pyr promoters. pTSROX3-encoded PyrR was expressed to about one-third of the total cell protein in E. coli strain SO408. In other experiments (not shown) the addition of uracil to the growth medium did not reduce the amount of pTSROX3-encoded PyrR produced in a different strain of E. coli. Thus, although the PyrR protein regulates its own expression in B. subtilis (3), such regulation was not observed in an E. coli background. Because transcriptional attenuation of pyr genes can be demonstrated with purified PyrR and E. coli RNA polymerase in vitro (9), we suggest that the failure to see such attenuation in vivo might reflect significant differences in the intracellular concentrations of the regulatory metabolites UMP and PRPP in the two species.

The procedure for purification of PyrR is relatively simple. The most effective step is ion exchange chromatography on Q-Sepharose. The prior steps were used primarily to remove non-protein contaminants so that the fractionation on Q-Sepharose would be more reproducible. The subsequent ammonium sulfate fractionation is useful to concentrate the protein and to remove some very minor contaminating proteins, but it frequently resulted in losses of activity and a reduced specific activity. This step can be omitted for many uses of the purified PyrR. At least four trace-contaminating proteins can be detected in the best preparations of PyrR on overloaded SDS-polyacrylamide gels (not shown); we estimate these preparations to be at least 98% pure. Specific UPRTase activities (using assay Method 1) of purified PyrR preparations have ranged from 6 to 11 µmol/min/mg at pH 9.

Physical Characterization of PyrR—Two preparations of purified PyrR were subjected to ESI-MS analysis. Three major components, comprising about 95% of the total protein, were resolved (results for one of the preparations are shown in Fig. 1). The mass of the most abundant component of each sample, approximately 70%, was 20,263 ± 2 Da. Another component, comprising approximately 10% of each preparation, had a mass of 131–132 Da smaller than the main component, which matches the change in mass expected for removal of the NH₂-terminal methionine that is known to be present on the bulk of PyrR produced in E. coli (3). The third major component, which comprised about 12% of the total protein, was 28 Da smaller than the most abundant component. This component and several other minor components were not identified.

The mass of the major components differed by 75 Da from the

FIG. 1. ESI-MS analysis of purified PyrR. Peak heights are proportional to the relative abundance of the peaks. The masses determined for each significant peak are printed above the peak.
TABLE I

Apparent native molecular weight of PyrR as a function of conditions of gel filtration chromatography on Sephadex G-150

| Concentration of PyrR loaded | Additions to elution buffer | M, of major peak |
|-----------------------------|-----------------------------|-----------------|
| mg/ml                       |                             |                 |
| 0.4                         | None                        | 75,000          |
| 5.0                         | None                        | 95,000          |
| 49.0                        | None                        | 100,000         |
| 0.4                         | 25 mM MgCl₂ + 2.5 mM UMP    | 85,000          |
| 0.4                         | 25 mM MgCl₂ + 2.5 mM PRPP   | 60,000          |

* A minor inactive protein peak at M, ≥ 300,000 was also detected.

mass predicted by the previously deduced amino acid sequence of PyrR (3). This discrepancy led us to re-examine the nucleotide sequence of pyrR. Two GC dinucleotide inversions were discovered which require codons 34 and 53 to be changed from encoding a serine (UGC) and arginine (CGC), respectively. These corrections to the PyrR sequence led to a calculated molecular mass (20,263) that matched exactly the value determined by ESI-MS. The corrected PyrR sequence contains one cysteinyl residue/monomer, whereas the previously deduced sequence predicted none. Titration of the sulfhydryl content of PyrR with 

A minor inactive protein peak at M, ≥ 300,000 was also detected.

The ultraviolet spectrum of PyrR at pH 7.5 yielded an extinction coefficient of 7.1 mM⁻¹ cm⁻¹ at 275 nm based on determination of its concentration by the Bradford method (15), which matched very well with an extinction coefficient at 275 nm of 7.5 mM⁻¹ cm⁻¹ calculated for the protein from a content of five tyrosine residues and no tryptophan residues predicted from the deduced sequence. Furthermore, this result indicates that the purified protein does not contain significant amounts of nucleotide or nucleic acid contaminants.

The native molecular weight of PyrR was determined by gel filtration chromatography at various protein concentrations and in the presence and absence of the ligands UMP or PRPP (Table I). In general, most of the PyrR migrated on gel filtration as a single, active peak whose apparent molecular weight was dependent on the concentration of PyrR loaded. Average molecular weights from 60,000 to 100,000 were observed; given the subunit molecular weight of 20,000, these values correspond to trimeric to pentamer states of aggregation. X-ray crystallographic analysis has identified two forms of PyrR, one dimeric and the other hexameric.2 We interpret the behavior of PyrR on gel filtration chromatography to indicate that the dimeric and hexameric forms of PyrR are in rapid equilibrium and that the average molecular weight values observed reflect shifts in that equilibrium toward the hexameric form as the PyrR concentration is increased. Mg²⁺ and UMP tend to favor the more aggregated form, whereas Mg²⁺ and PRPP favor the less aggregated form of PyrR, but these effects are small, and in no case is the protein fully converted to one state of aggregation. In the absence of stabilizing ligands PyrR has some tendency to form highly aggregated inactive species. A much more pronounced ability of substrates to affect the state of aggregation of the UPRTase from E. coli was reported by Jensen and Mygind (18), who found that substrates converted the enzyme from a low molecular weight form, probably a dimer, to a high molecular weight form, probably a hexamer. B. subtilis PyrR shares with E. coli UPRTase the ability to be stabilized and activated by prior incubation with Mg²⁺ and PRPP (see below), but we did not find evidence for promotion of aggregation of PyrR by this substrate.

UPRTase Activity of PyrR—The UPRTase activity of PyrR was first identified from the ability of the pyrR gene to complement the upp mutation in E. coli SO408 and assays of UPRTase activity in SO408 cells bearing a pyrR-encoding plasmid (3). This activity was confirmed by the very high levels of UPRTase activity in cells in which PyrR was overexpressed and by the increase in specific activity of UPRTase as PyrR was purified to homogeneity. Assays of the UPRTase activity of PyrR in crude extracts were initiated by the addition of PRPP because the presence of many other PRPP-consuming enzymes made preincubation of the crude extract with PRPP undesirable. When PyrR was purified, however, the high dilution of the protein needed to bring the assay into the linear range required preincubation of PyrR with Mg²⁺ ions and PRPP to obtain assays that were linear with time and to avoid inactivation of the enzyme at high dilution. This approach was suggested to us by the studies of Jensen and Mygind (18), who showed that the UPRTase from E. coli is converted to a more highly aggregated and more active form by incubation with Mg²⁺ and PRPP. In the case of PyrR, stabilization of the UPRTase activity by Mg²⁺ and PRPP was especially necessary when the protein was diluted and incubated at 37 °C instead of 0 °C. Dilution of PyrR into buffer containing 1 mg/ml BSA was also necessary to prevent losses of UPRTase activity.

The values for the maximal velocity and the Michaelis constants for PRPP and uracil for the UPRTase reaction catalyzed by PyrR were determined at 5 mM MgCl₂ and 37 °C in the pH range from 7.7 to 9.7 (Fig. 2). (We were unable to determine kinetic constants accurately at lower pH values because of severe substrate inhibition by uracil.) The UPRTase activity of PyrR consistently displayed a Ping Pong kinetic pattern. Maximal activity at saturating substrate concentrations was at pH 8.2. The Michaelis constant for uracil was very dependent on the reaction pH, rising from around 100 μM at pH 9.2–9.7 to about 1 mM at pH 7.7. The Michaelis constant for PRPP was also somewhat dependent on pH; the minimal value of 70 μM was observed at pH 8.7 with larger values observed at both higher and lower pH. The K values for PyrR-catalyzed UPRTase are in contrast to the values of about 50 μM for PRPP and 2 μM for uracil observed with the B. caldolyticus upp-encoded UPRTase at pH 8.6 (19). We suggest that these kinetic differences between the pyrR-encoded UPRTase and the upp-encoded UPRTase, which has much greater sequence similarity to other bacterial UPRTases (7), explain why the upp-encoded enzyme is the physiologically dominant UPRTase in B. subtilis (7); the latter enzyme has a much smaller Michaelis constant for uracil and is thus much more effective in uracil salvage.

RNA Binding to PyrR—The binding of 32P-labeled RNA to PyrR was measured by an electrophoretic gel mobility shift assay as described under “Experimental Procedures.” In most cases purified PyrR was used; the radioactive oligonucleotide used for most of the characterization of binding was an 80-nucleotide segment corresponding to residues +682 through +761 from the conserved region of the pyrR-pyrP intercistronic region, i.e. the anti-antiterminator of the second attenuation region (3, 5), which had been shown in preliminary studies to bind well to PyrR. The specificity of the interaction between PyrR and pyr mRNA was tested in two ways. First, to demonstrate that the RNA was bound specifically by PyrR, gel shift experiments were performed using the 80-nucleotide pyr
RNA and crude extracts from either *E. coli* SØ408/pTSROX3, which overexpresses PyrR, or *E. coli* SØ408/pUC18, which carries the vector plasmid only. The crude extract containing overexpressed PyrR clearly contained a protein that binds RNA; increasing amounts of this extract increased the amount of RNA bound (Fig. 3). In contrast, the crude extract from cells that contained the vector only contained no protein that bound detectably to RNA; at the highest concentrations of such extracts tested some degradation of the RNA was evident. These results indicate that the PyrR protein binds to RNA and rules out the possibility that an impurity in the PyrR preparation binds to the RNA instead.

To demonstrate that PyrR binds specifically to *pyr* mRNA, the following radioactive RNAs were tested with purified PyrR: the 80-nucleotide segment of *pyr* RNA described above, an 18 S rRNA transcript, an actin mRNA transcript, and RNA transcribed from the multiple cloning site of pSP72 (Promega) linearized with *Hind*III. PyrR bound only to the *pyr* mRNA (data not shown). A more rigorous test of specificity was provided by examining the binding of PyrR to segments of *pyr* mRNA from the same attenuation region corresponding to the antiterminator stem-loop (nucleotides +772 to +809), and the antisense sequence of RNA derived from the same segment of the attenuator as the 80-nucleotide RNA for which the sense strand was shown above to bind well to PyrR. None of these RNA species was bound by PyrR under conditions in which the 80-nucleotide RNA that included the anti-antiterminator sequence bound very well (Fig. 4). Because all three of the control RNAs are derived from *B. subtilis* *pyr* sequences and all three are predicted to form stem-loop structures, the failure of PyrR to bind detectably to these structures is very strong evidence for its specificity for a defined *pyr* mRNA sequence. Moreover, the sequence to which PyrR bound included the sequence that is conserved in all three *pyr* attenuation regions (3) and is positioned such that binding of the protein would disrupt formation of the antiterminator stem-loop, as predicted by our model (3, 20) for attenuation control of the *pyr* operon. More detailed studies of the specificity of binding of PyrR to *pyr* mRNAs from each of the three attenuation regions and determination of specific sequence and secondary structural requirements for RNA recognition are in progress in our laboratory.

An approximate *K* for the PyrR-*pyr* mRNA interaction was determined in gel shift experiments in which the RNA concentration was held constant and the PyrR concentration was varied, using triplicate determinations of each point (Fig. 5). At saturating UMP the *K* determined was 10.2 ± 0.5 nM; when UMP was omitted from the binding mixtures the *K* was 216 ± 17 nM. This 20-fold difference in apparent binding affinity of PyrR for *pyr* mRNA in the presence versus the absence of UMP may be sufficiently large to account for the physiological regulation of *pyr* attenuation which is known to occur (3), although it is appreciably smaller than the differences in affinity of well...
characterized repressor proteins for operator DNA in the presence and absence of corepressors. It is possible that the conditions used for measuring binding by gel mobility shift do not reproduce the binding conditions in vivo adequately. We have tested other gel mobility shift conditions in which tight, specific binding occurred, but no dependence of RNA binding on the presence of UMP could be demonstrated. Thus, the UMP dependence of PyrR binding to RNA is quite sensitive to the experimental conditions, and the current observations may not give a reliable estimate of the degree of this dependence in vivo.

The concentration of UMP which gives half-maximal stimulation of PyrR binding to pyr mRNA was estimated by gel shift experiments in which the PyrR and RNA concentrations were held constant and the concentration of UMP was varied from 0 to 500 μM. On the basis of the curves shown in Fig. 5, 80 nM PyrR was chosen to give a substantial difference between the fraction of bound RNA in the presence versus the absence of UMP. Fig. 6 demonstrates that 5.8 ± 0.8 μM UMP gave half-maximal stimulation of PyrR binding. This value is comparable to the concentration of 2.5 μM UMP which was shown previously to give half-maximal stimulation of transcriptional termination with a pyr template by PyrR in vitro (9).

Relationships between PyrR and other RNA-binding Attenuation Proteins—Although our studies have concentrated on PyrR from B. subtilis, it has become clear that PyrR homologs are found in many species of bacteria in which they probably also regulate pyr gene expression; examples include B. caldolyticus (8), Enterococcus faecalis (21), Lactobacillus plantarum (22), and Lactococcus lactis (23). Recently, a Thermus species has been shown to encode a PyrR that probably binds to pyr mRNA but acts as a translational repressor (24). Genes with strong sequence similarity to pyrR have been found in two other species, but it is less clear whether they function as RNA-binding regulatory proteins in these cases (25, 26).

PyrR is a member of a small group of proteins that regulate gene expression by binding to mRNA and affecting transcriptional termination at a downstream site (27), but we suggest that many more such proteins remain to be discovered. The B. subtilis trp RNA-binding attenuation protein, TRAP, is the only well characterized example of a regulatory protein that is functionally quite similar to PyrR (28–31). Like PyrR, TRAP brings about transcriptional termination by binding to a specific site on mRNA and preventing formation of an antitermination hairpin, which permits formation of a downstream transcription terminator (28, 29). However, TRAP has no known enzymatic activity. Its quaternary structure (30) and the nature of RNA sequences recognized by TRAP are very different from PyrR (30, 31).² A somewhat different class of mRNA-binding regulatory proteins comprises proteins, such as E. coli BglG (32) and B. subtilis SaacT and SaacY (33–36), which act by binding to a transcription terminator that precedes the genes to be regulated and suppressing termination. PyrR and TRAP binding to RNA is regulated by the end products of the operons they control, i.e. by UMP and tryptophan, respectively, whereas the ability of BglG, SaacT, and SaacY to bind to RNA is regulated by reversible phosphorylation. Not only do these systems present novel mechanisms for the control of gene expression in bacteria, but we believe they provide favorable objects for the detailed study of protein-RNA recognition in general.

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