Kinetic Studies of the Uracil Phosphoribosyltransferase Reaction Catalyzed by the Bacillus subtilis Pyrimidine Attenuation Regulatory Protein PyrR*

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The PyrR protein from Bacillus subtilis and many other bacteria is a bifunctional protein. Its primary function is the regulation of expression of pyrimidine biosynthetic (pyr) genes by binding to specific sites on pyr mRNA in a uridine nucleotide-dependent manner and altering the folding of downstream RNA to promote termination of transcription. PyrR also catalyzes the uracil phosphoribosyltransferase (UPRTase) reaction even though it bears little amino acid sequence similarity to other bacterial UPRTases. The PyrR-catalyzed UPRTase reaction obeyed a Ping Pong steady state kinetic pattern even though it bears little amino acid sequence similarity to other bacterial UPRTases. The PyrR-catalyzed UPRTase reaction obeyed a Ping Pong steady state kinetic pattern even though it bears little amino acid sequence similarity to other bacterial UPRTases. This mechanism was supported by the following experimental observations. The reverse reaction was extremely slow with a catalytic rate constant for PyrR, which was solved at high resolution by Tomchick et al. (9), demonstrated that the PyrR structure is very similar to other Type I phosphoribosyltransferases (10). We have speculated that PyrR arose from evolution of an ancestral phosphoribosyltransferase, possibly a hypoxanthine guanine phosphoribosyltransferase, which PyrR most resembles in sequence and tertiary structure, by gaining the ability to bind to a specific RNA structure (9).

A remarkable property of PyrR, first discovered by Ghim and Neuhard in studies of PyrR from the thermophile Bacillus caldolyticus (7), is that it also catalyzes the uracil phosphoribosyltransferase (UPRTase) reaction. This finding was unexpected because the deduced amino acid sequences of PyrR proteins from various bacteria bear no significant sequence similarity outside of a short sequence in the active site to the sequences of previously characterized bacterial UPRTases, which are encoded by upp genes (1). Nonetheless, purified B. subtilis PyrR, when assayed under optimal conditions, has an activity specific activity comparable with purified bacterial upp-encoded UPRTases (8). Furthermore, the three-dimensional structure of B. subtilis PyrR, which was solved at high resolution by Tomchick et al. (9), demonstrated that the PyrR structure is very similar to other Type I phosphoribosyltransferases (10). We have speculated that PyrR arose from evolution of an ancestral phosphoribosyltransferase, possibly a hypoxanthine guanine phosphoribosyltransferase, which PyrR most resembles in sequence and tertiary structure, by gaining the ability to bind to a specific RNA structure (9).

This communication presents a kinetic study of the UPRTase reaction catalyzed by PyrR. We have sought to determine the relationship between this reaction and the UPRTase reaction catalyzed by upp-encoded UPRTases and by Type I phosphoribosyltransferases in general. Our results indicate that PyrR is a rather typical phosphoribosyltransferase. We propose a kinetic model for the UPRTase reaction that explains how it displays a Ping Pong steady state kinetic pattern but does not function via a phosphoribosyl-enzyme intermediate. Our model provides a mechanistic resolution to a number of apparently contradictory kinetic studies of other phosphoribosyltransferases in the literature. Some observations on the relationship between the UPRTase activity of PyrR and its function as an mRNA binding attenuation regulatory protein are also presented.

**EXPERIMENTAL PROCEDURES**

All procedures are described in greater detail in Grabner (11). Materials—B. subtilis PyrR was purified by the procedure of Turner et al. (8) with the following modifications. The supernatant fluid from the streptomycin precipitation step was applied directly to the QAE-

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1 The abbreviations used are: UPRTase, uracil phosphoribosyltransferase (PRTase); PRPP, phosphoribosylpyrophosphate.
Sophorose column without ammonium sulfate precipitation or dialysis, the column was washed with 400 ml of Buffer B (100 mM Tris acetate, 10 mM Na\textsuperscript{+}-phosphate, pH 7.0 (8) containing 150 mM NaCl instead of 100 mM NaCl, and PyrR was eluted with an 800-mI linear gradient from 150 to 250 mM NaCl in Buffer B. The purified PyrR fractions were concentrated with powdered ammonium sulfate to 80% saturation and dialyzed three times against 2 liters of 100 mM Tris acetate, 10 mM potassium acetate, 10% glycerol, pH 7.5, at 4 °C.

PRPP, uracil, UMP, UDP, and UTP were commercial products of Sigma-Aldrich. The concentrations of PRPP solutions was determined spectrophotometrically. The \([32P]PPi-PRPP exchange reaction was assayed by incubating 2 mM \([32P]PPi\) with 50 mM PRPP (1000 Ci/mmol) for 1 h. Uracil, when added, was at 1 mM. Samples (5 \(\mu\)l) were removed at various time intervals less than 1 min to allow the enzyme to ensure linearity of product formation (time) and spotted and dried on DEAE-cellulose paper, which was washed to remove unreacted \([14C]\)adenine and analyzed by thin layer chromatography polyethyleneimine-cellulose plates. Impurities were undetected (2%) and 11% (unknown 16%). The \([32P]UMP\) was diluted with pure nonradioactive UMP of known concentration for use in equilibrium dialysis, correcting the specific radioactivity for its 78% purity. The \([32P]PRPP\) was synthesized from [\(\alpha-32P\)]UTP and 5-phosphoribosylamine (5 mM \(\mu\)Ci/mmol, ICN Biochemicals) using 10 \(\mu\)g (0.36 units) of purified human PRPP synthetase isozyme II (12) in 50 mM KH\textsubscript{2}PO\textsubscript{4}, 50 mM triethanolamine, 0.1 mM MgCl\textsubscript{2} (in future experiments 2 mM), and 150 mM NaCl instead of pH 7.0 (8) containing 150 mM NaCl instead of 100 mM NaCl, and PyrR was eluted with an 800-mI linear gradient from 150 to 250 mM NaCl in Buffer B. The purified PyrR fractions were concentrated with powdered ammonium sulfate to 80% saturation and dialyzed three times against 2 liters of 100 mM Tris acetate, 10 mM potassium acetate, 10% glycerol, pH 7.5, at 4 °C.

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Assays for UPRTase and Exchange Reactions—The UPRTase forward (uracil + PRPP \(\rightarrow\) PPi + UMP) reaction was assayed by Method 2 previously described (8). The \([32P]\)uracil-UMP exchange reaction was assayed by Method 1, except that \([32P]PRPP\) was omitted from the reactions. The UPRTase reverse (UMP + PPi \(\rightarrow\) uracil + PRPP) reaction was assayed by enzymatic conversion of \([32P]PPi\) to \([32P]UPRTase catalyzed by PyrR

\[ \text{E} + \text{PRPP} \leftrightarrow \text{phosphoribosyl-E} + \text{PPi} \]  
(\text{Eq. 1})

\[ \text{phosphoribosyl-E} + \text{uracil} \rightarrow \text{E} + \text{UMP} \]  
(\text{Eq. 2})

Such a mechanism predicts the ability of the enzyme to catalyze two independent exchange reactions at rates equal to or greater than the overall reaction rate, namely \([14C]\)uracil-UMP exchange and \([32P]PRPP-PRP\) exchange. We examined the ability of PyrR to catalyze these reactions using the methods described under “Experimental Procedures.” No exchange reaction was detected. No exchange of \([14C]\)uracil into UMP could be detected using up to 162 \(\mu\)g of PyrR per reaction; in contrast, when 100 mM PRPP was included in the reaction mixture \([14C]\)uracil was incorporated into UMP at a readily detectable rate (0.024 nmol/min) with only 0.0162 \(\mu\)g of PyrR added. In attempts to observe \([32P]PRPP\)-PRP exchange up to 244 \(\mu\)g of PyrR was added to each reaction mixture, but no exchange of \([32P]PPi\) into PRPP was observed. However, in this case formation of \([32P]PRPP\) from \([32P]PPi\) and uracil was also not detectable, so direct proof that the PyrR was active was not obtained. However, PyrR treated in the same manner was highly active in assays of the forward UPRTase reaction. As will be shown below, the catalysis of the reverse reaction by
PyrR is exceedingly slow; we believe this accounts for the failure to observe it under conditions used in the \[^{32}P\]PPi-PRP exchange assay.

An Ordered Bi Bi Kinetic Model with a Kinetically Irreversible Conformational Change Can Account for the Kinetic Behavior of PyrR-catalyzed UPRTase

The cardinal property of Ping Pong kinetics that accounts for the parallel lines observed in double reciprocal plots is not the formation of a covalent enzyme intermediate but the imposition of a kinetically irreversible step between the binding of the first substrate and the binding of the second substrate. In the mechanism shown above that irreversible step is the dissociation of the first product, PPi, into a solution in which its initial concentration is zero. We therefore considered whether one of several Ordered Bi Bi kinetic mechanisms in which a kinetically irreversible conformational change occurs during catalysis could account for our kinetic observations. In devising these models we took into consideration the fact that several Type I phosphoribosyltransferases have been experimentally demonstrated to undergo large conformational changes after the binding of PRPP (16). We reasoned that if such a conformational change occurred in PyrR after the binding of PRPP and this step were kinetically irreversible, such a mechanism would give rise to Ping Pong kinetic behavior (by “kinetically irreversible” we mean that the forward direction is very much faster than the reverse direction and that the reverse reaction, if it occurs at all, is very much slower than the other kinetic steps of the mechanism.) This presumption was verified by derivation of the steady state rate equations for the four kinetic mechanisms shown in Fig. 2 by the method of King and Altman (17). These four mechanisms are all Ordered Bi Bi mechanisms in which PRPP is the first substrate to bind and UMP is the last product to dissociate from the enzyme. In all cases the kinetically irreversible step is postulated to be a conformational change that occurs after the binding of PRPP but before the binding of uracil. The mechanisms differ in the nature of the step at which the enzyme returns to the unliganded conformation. The steady state equation for the reaction in the absence of products for all four mechanisms is the same and is that found for any bisubstrate Sequential kinetic mechanism.

\[
\frac{v}{V_{\text{max}}} = \frac{[A][B]}{K_a K_{\text{cat}} [A] + K_a [B] + [A][B]} \quad \text{(Eq. 3)}
\]

However, if one assumes that the rate constant for the reverse of the conformational change, \(k_{-2}\), approaches zero, the equation simplifies to that obtained for Ping Pong mechanisms.

\[
\frac{v}{V_{\text{max}}} = \frac{[A][B]}{K_{a}[A] + K_{a}[B] + [A][B]} \quad \text{(Eq. 4)}
\]
Thus, all four mechanisms predict the absence of a slope effect in double reciprocal plots. However, if the product terms are included, the four mechanisms differ in the product inhibition patterns they predict (Table I). Complete details of the derivations and deduction of kinetic patterns are presented in Grabner (11).

Product Inhibition Studies Favor Model 1—Product inhibition studies of PyrR-catalyzed forward reaction (PRPP + uracil → UMP + PP) were conducted and are summarized in Table II. The pattern of inhibition could be unambiguously assigned on the basis of the quality of the statistical fit of the data to the appropriate equations in all cases except for inhibition by PPi as a function of uracil concentration. In that case uncompetitive inhibition could be excluded, but the fit of the data to competitive and to noncompetitive patterns was essentially equivalent. Table I lists the patterns of product inhibition predicted for Ping Pong, Random Bi Bi, Ordered Bi Bi (18), and the four irreversible conformational change models described in Fig. 2. It can be seen that, even with ambiguity in the pattern of PPi, inhibition versus uracil saturation, our observations are consistent only with Model 1 of Fig. 2.

Also shown in Table II are the results of inhibition studies performed with UDP and UTP. These nucleotides were investigated because it has been shown that they, like UMP, affect PRPP binding was also hyperbolic and fit a dissociation equation for a Ping Pong mechanism and yielded values of 0.0045 ± 0.001 μmol/min/mg for Vmax (kcat = 1.5 ± 0.3 × 10⁻³ s⁻¹), 130 ± 37 μM for the Km for UMP, and 1000 ± 390 μM for the Km for PPi. By contrast, the kcat for the forward reaction at the same pH (8.7) was 5.1 s⁻¹, 3300 times higher than for the reverse reaction.

Studies of the Binding of Substrates to PyrR by Equilibrium Dialysis—A prediction of the Ordered Bi Bi kinetic model we have proposed that accounts for the Ping Pong kinetics we observed is that only the first substrate to bind, PRPP, and the last product to dissociate, UMP, should bind to the free enzyme. We were also interested in examining the binding of these substrates and of UDP, all of which have been shown to affect the apparent affinity of PyrR for RNA (2). Binding of commercial [α-³²P]UTP, [¹⁴C]uracil, and [³²P]PPP, synthesized as described under “Experimental Procedures,” to highly purified PyrR was studied by equilibrium dialysis. Neither [¹⁴C]uracil nor [³²P]PPP, bound detectably to 100 μM PyrR at an initial concentration as high as 1.5 mM, but [³²P]UMP and [³²P]PPPP bound well (Fig. 3, A and B). [³²P]PPPP binding was well described by a simple hyperbolic curve corresponding to a dissociation constant of 27 ± 3 μM. [³²P]PRPP binding was also hyperbolic and fit a dissociation constant of 18 ± 2.4 μM. Binding at saturating ligand extrapolated to 0.56 ± 0.02 mol of UMP per mol of PyrR subunit and 0.79 ± 0.03 mol of PRPP per mol of PyrR. This stoichiometry of binding varied somewhat from experiment to experiment but was generally less than 1 mol of ligand per mol of PyrR. The reason for this is not known. The UPTase activity of PyrR was shown to be constant over the time required for dialysis. Molecular sieving analysis by high performance liquid chromatography indicates that PyrR is present entirely as a hexamer at the concentration used for equilibrium dialysis (3), although it is possible that some more highly aggregated forms that fail to bind substrates also form during the 40–48-h dialysis. Binding of PRPP and UMP to the free enzyme and failure of uracil and PPi to bind are consistent with the predictions of an Ordered Bi Bi mechanism in which PRPP binds first and UMP dissociates ceased when only a small percentage of the substrates was consumed. The amount of products formed was severalfold greater than and did not depend on the concentration of PyrR, so the reverse reaction was not limited to a single “burst.” Despite these difficulties, we were able to conduct a study of the dependence of the rate of the reverse reaction on substrate concentration under conditions where the reactions could be shown to be linear with both time and the amount of PyrR used. The data fit best to the equation for a Ping Pong mechanism and yielded values of 0.0045 ± 0.001 μmol/min/mg for Vmax (kcat = 1.5 ± 0.3 × 10⁻³ s⁻¹), 130 ± 37 μM for the Km for UMP, and 1000 ± 390 μM for the Km for PPi. By contrast, the kcat for the forward reaction at the same pH (8.7) was 5.1 s⁻¹, 3300 times higher than for the reverse reaction.

| Mechanism     | Product inhibitor | PRPP | Uracil |
|---------------|------------------|------|-------|
| Ping Pong     | PP, UMP          | Noncompetitive | Competitive |
| Random Bi Bi  | PP, UMP          | Competitive | Noncompetitive |
| Ordered Bi Bi | PP, UMP          | Noncompetitive | Noncompetitive |
| Model 1       | PP, UMP          | Uncompetitive | Noncompetitive |
| Model 2       | PP, UMP          | Competitive | Noncompetitive |
| Model 3       | PP, UMP          | Uncompetitive | Noncompetitive |
| Model 4       | PP, UMP          | Competitive | Uncompetitive |
| Observed      | PP, UMP          | Competitive or noncompetitive | Noncompetitive |

* Predicted from derived equations; see Grabner (11).
last and with each of the four models described in Fig. 2. Such binding is inconsistent with a Ping Pong mechanism or a Random Sequential mechanism.

Binding of [α-32P]UTP was too weak to be well characterized by our equilibrium dialysis conditions (Fig. 3C). Binding of UTP was clearly above background, but the dissociation constant could only be characterized as greater than 800 μM.

Equilibrium dialysis studies of the binding of [32P]UMP and [α-32P]UTP were repeated with 100 μM each of PyrR and a 36-nucleotide pyr RNA that was known from the studies of

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**Table II**

| Inhibitor | Varied substrate | Inhibition pattern | Inhibition constant (μM) |
|-----------|------------------|--------------------|-------------------------|
| UMP       | PRPP             | Competitive        | 122 ± 12                |
| UMP       | Uracil           | Uncompetitive      | 149 ± 15                |
| UDP       | PRPP             | Competitive        | 232 ± 28                |
| UDP       | Uracil           | Uncompetitive      | 253 ± 28                |
| UTP       | PRPP             | Competitive        | 1066 ± 132              |
| UTP       | Uracil           | Uncompetitive      | 924 ± 82                |
| Pi        | PRPP             | Uncompetitive      | 2200 ± 500              |
| Pi        | Uracil           | Competitive or Noncompetitive | 1800 ± 500 |

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**Fig. 3.** Binding of substrates and UTP to PyrR, as determined by equilibrium dialysis. A, binding of [32P]PRPP. B, binding of [32P]UMP. C, binding of [α-32P]UTP. In all experiments data from dialyzing in and dialyzing out protocols were combined. In panel C the data from two binding studies were combined.
The binding of PRPP and UMP used for estimates of the conformation of the RNA had no statistically significant effect on the amount of dissociation constant in the nanomolar range. The presence of either nucleotide bound to PyrR or their dissociation constants.

### DISCUSSION

**Kinetic Mechanism of the PyrR-catalyzed UPRTase Reaction**—Kinetic and binding constants for the PyrR-catalyzed UPRTase reaction are summarized in Table III. Although we were not able to conduct a product inhibition study of the reverse reaction and, thus, determine a complete set of steady state kinetic constants, the results allow a mechanism to be suggested. We propose here a kinetic mechanism for the UPRTase reaction catalyzed by *B. subtilis* PyrR that is basically an Ordered Bi Bi mechanism with PRPP binding first and UMP dissociating last. However, to account for the Ping Pong kinetic patterns consistently observed with this enzyme, we have postulated in addition a kinetically irreversible conformational change following the binding of PRPP. This postulate was prompted by the abundant evidence in the literature for conformational changes that accompany or follow binding of PRPP to several Type I PRTases (16, 19–22). Model 1, which fits the observed patterns of product inhibition best, also postulates that PyrR returns to the unliganded conformation before the dissociation of UMP. The following experimental observations are consistent with the proposed kinetic mechanism.

1. Exchange reactions predicted by a classical Ping Pong mechanism involving formation of a phosphoribosyl-enzyme intermediate were not detected.
2. The reverse of the PyrR-catalyzed UPRTase reaction was extremely slow, 3300 times slower than the forward reaction. This observation is consistent with a very slow reversal of the E(PRPP) conformation to the E(U) conformation, but of course it does not specifically identify this step as rate-limiting for the reverse reaction.
3. PRPP and UMP bound with high affinity to free PyrR, but uracil and PP, did not bind at all at concentrations up to 0.8 mM. This result is consistent with an Ordered Bi Bi mechanism.
4. The kinetic patterns of product inhibition were inconsistent with all of the other mechanisms considered (Table 1).
5. The experimentally determined value for the equilibrium constant for the UPRTase reaction, which is 1.2 to 1.5 \( \times 10^4 \) at pH 8.5 and 37 °C (29), agreed within a factor of 2 with the value calculated from the Haldane relationship for our proposed mechanism,

\[
K_{eq} = \frac{k_{cat}^{(forward)}[K_p^{PP},[K_3^{UMP}]}{k_{cat}^{(reverse)}[K_3^{uracil},[K_p^{PRPP}]} = 3 \times 10^4 \quad (Eq. 5)
\]

We consider this agreement to be acceptable given that the value for the Michaelis constant for PP, could not be determined very accurately and that the dissociation constants for binding of PRPP and UMP used for estimates of the corresponding inhibition constants were determined at pH 7.5 and 0 °C. As is always true of steady state kinetic analysis, consistency of the observations with a given kinetic model does not establish that model conclusively. A complete study of the PyrR-catalyzed UPRTase reaction using pre-steady state methods would be needed to test our model further.

**Implications for the Mechanism of Other PRTases**—The Type I phosphoribosyltransferases all catalyze the transfer of a phosphoribosyl group from PRPP to a nitrogenous nucleophilic acceptor with inversion of configuration about C-1 of the ribose moiety, and they all share highly homologous three-dimensional structures (16). It is very likely that the mechanism of catalysis of these PRTases is fundamentally the same. However, both Ping Pong (23–27) and Sequential (28–31) steady state kinetic patterns have been frequently reported. The human HGPRTase has even been reported to display Ping Pong kinetics under some experimental conditions and Sequential kinetics under others (32). A classical Ping Pong mechanism predicts the existence of a free phosphoribosyl-enzyme intermediate and capacity to catalyze exchange reactions between reactant pairs in the absence of co-substrates. These properties have been reported for various PRTases (27, 33), but on further scrutiny the conclusions were invalidated (28, 34, 35). In no case has persuasive evidence for a classic Ping Pong mechanism been put forward for a Type I PRTase. How, then, are the frequent Ping Pong kinetic patterns to be explained? We believe that our irreversible conformational change model for PyrR-catalyzed UPRTase can provide a general solution to this question.

We cited evidence above from both structural and kinetic studies that some PRTases undergo large conformational changes upon the binding of PRPP. In the case of *Escherichia coli* glutamine PRPP amidotransferase this conformational change was shown to be relatively slow (19). In a very detailed kinetic study, Xu et al. (31) identify the product release step of human HGPRTase, which follows an Order Bi Bi kinetic mechanism, to be rate-limiting in both directions. They suggested that these steps were slow because of rate-limiting conformational changes that accompany or follow them. We propose that binding of PRPP to the free enzyme followed by a conformational change and then by binding of the second substrate is a general property of the Type I PRTases. Furthermore, we suggest that the reversibility of this conformational change determines whether the enzyme will display a Sequential or a Ping Pong kinetic pattern, with the latter resulting from very slow reversal. If this view is correct, we would predict that PRTases displaying Ping Pong kinetic patterns will be either kinetically irreversible or show much slower rates of their reverse reaction than of the forward (PRPP-dependent) reaction. The ratio of catalytic rates constant for the forward versus for the reverse reaction is about 100 for *upp*-encoded UPRTase from *E. coli*, for example, which follows a Sequential kinetic pattern (29). Thus, we propose that this ratio would be 1000-fold or greater for PRTases that obey Ping Pong kinetics. This prediction is relatively easy to test. Note that it is not necessary that a given PRTase displaying Ping Pong kinetics obey Model 1; many variants can be imagined that predict the same pattern.

**Implications Concerning the Physiological Functions of PyrR**—Even though it bears little amino acid sequence similarity to the UPRTases encoded by bacterial *upp* genes, PyrR has catalytic properties that are typical of most PRTases. The only significant exception to this was reported previously (8): PyrR has \( K_m \) values for uracil at physiological pH values that are more than 2 orders of magnitude larger than observed with bacterial UPRTases. Thus, PyrR would be relatively ineffective in uracil salvage. However, *B. subtilis* (36) and *B. caldolyticus* (7) *pyr* genes have been shown to encode functional UPRTases in vivo.

Two of the most important effectors of PyrR binding to *pyr*
mRNA, UMP and PRPP, are a product and a substrate, respectively, of its URPTase activity. Our results are consistent with the proposal that these molecules affect PyrR binding to RNA by binding to the URPTase active site, although they cannot conclusively prove this. The dissociation constants for UMP and PRPP are reasonably close to the concentrations at which they exert half-maximal effects on RNA binding (2) or transcription termination (6), especially given the differences in reaction conditions used in the different studies. PRPP antagonizes the effects of UMP on transcription termination (6), which indicates competition for the same site, a result also obtained in our kinetic studies. UTP exerts similar effects as UMP on transcription termination (6) and binding of PyrR to RNA (2), but 10–100-fold higher concentrations of UTP are required. The kinetic pattern of inhibition of URPTase activity by UTP and its 10-fold larger inhibition constant supports the idea that UTP also regulates the attenuation functions of PyrR by binding to the UMP portion of the URPTase active site. Binding of UTP to PyrR was appreciably weaker than we expected, however.

Because UMP and PRPP affect the affinity of PyrR for pyr mRNA, one might expect a reciprocal effect, i.e. alteration of binding of UMP or PRPP by RNA in equilibrium dialysis experiments or effects of RNA on the kinetics of URPTase activity. No such effects were observed either in the dialysis experiments or in studies of UMP and PRPP saturation of PyrR in URPTase assays at 37 °C (Ref. 11 and data not shown). However, there was no way to prove that the pyr RNA was bound to PyrR under the conditions of equilibrium dialysis, where the concentration of PyrR was much higher than in RNA binding studies, or at the assay temperature of 37 °C (the binding studies were conducted at 0 °C). A more direct analysis of the interactions between the URPTase active site and the RNA binding site of PyrR must await the determination of a high resolution structure of a PyrR-RNA complex. Attempts to achieve this goal are in progress.

Acknowledgments—We acknowledge Michael Becker for providing the purified adenine phosphoribosyltransferase used in assays of the reverse reaction and Bryce Flapp for the Kinsim computer program used in analysis of kinetic data.

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