The σ^70 Transcription Factor TyrR Has Zinc-Stimulated Phosphatase Activity That Is Inhibited by ATP and Tyrosine†

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Received 19 August 1999/Accepted 23 November 1999

The TyrR protein of Escherichia coli (513 amino acid residues) is the chief transcriptional regulator of a group of genes that are essential for aromatic amino acid biosynthesis and transport. The TyrR protein can function either as a repressor or as an activator. The central region of the TyrR protein (residues 207 to 425) is similar to corresponding polypeptide segments of the NtrC protein superfamily. Like the NtrC protein, TyrR has intrinsic ATPase activity. Here, we report that TyrR possesses phosphatase activity. This activity is subject to inhibition by l-tyrosine and its analogues and by ATP and ATP analogues. Zinc ion (2 mM) stimulated the phosphatase activity of the TyrR protein by a factor of 57. The phosphatase-active site of TyrR was localized to a 31-kDa domain (residues 191 to 467) of the protein. However, mutational alteration of distant amino acid residues at both the N terminus and the C terminus of TyrR altered the phosphatase activity. Haemophilus influenzae TyrR (318 amino acid residues), a protein with a high degree of sequence similarity to the C terminus of the E. coli TyrR protein, exhibited a phosphatase activity similar to that of E. coli TyrR.

The TyrR protein of Escherichia coli K-12 regulates the transcription of a group of genes involved in aromatic amino acid biosynthesis and transport (28). Transcriptional regulation by TyrR can be either negative or positive. In those cases where the TyrR protein functions as a repressor, the affected genes are aroF, aroL, tyrB, aroP, aroH, and tyrR. In the case of aroP, transcription is inhibited by the TyrR-mediated formation of a nonproductive complex between RNA polymerase and an overlapping divergent promoter (30). For aroL, TyrR-mediated looping of DNA is thought to occur (18). TyrR functions as an activator of the mtr gene (12, 26) and the gpl gene (27). For the gpl gene, TyrR can either repress or activate, depending on whether phenylalanine (activation response) or tyrosine (repression response) is provided (15). TyrR binds specifically to a group of 22-bp DNA target sequences, termed strong or weak TyrR boxes, that are situated within or immediately upstream of the regulated promoters. The binding of TyrR to DNA is ligand mediated. Tyrosine increases the ability of TyrR to bind to weak boxes, but only when ATP is present and when there is an adjacent strong box. The precise mechanism by which TyrR mediates repression and activation of gene expression is unknown.

TyrR contains 513 amino acid residues (8, 34). The protein is predominantly homodimeric in solution, but it can self-associate to give rise to hexameric structures (34). In the gpl system, the formation of higher-order aggregates between TyrR dimers occurs in the presence of DNA containing multiple TyrR boxes (5).

Upon limited trypsin digestion, the TyrR protein gives rise to two trypsin-resistant subfragments of 22 and 31 kDa. The smaller fragment contains residues 1 to 190, while the larger fragment contains residues 191 to 467. The second domain of TyrR (residues 207 to 425) displays a high degree of sequence similarity to the central region of the NtrC protein superfamily, a group of proteins that specifically activate genes transcribed by the σ^54 form of RNA polymerase (30).

The σ^54-specific regulatory proteins that bear sequence similarity to TyrR fall into two classes. The first class (e.g., NtrC, DctD, AlgB, etc.) belong to so-called two-component systems (21). The activity of this class of transcriptional regulators is dependent upon their phosphorylation and dephosphorylation by a second sensor protein. The second class (e.g., FhlA, etc.) is structurally homologous to the first class in the central and C-terminal domains but is not known to undergo phosphorylation (7). Transcriptional regulation by the latter group of proteins may therefore involve direct activation through the binding of a low-molecular-weight ligand.

Each of the genes controlled by TyrR is transcribed by the σ^70 form of RNA polymerase (23). Transcriptional activation by TyrR is thought to involve direct contact between TyrR and the α subunit of RNA polymerase (19). Yang et al. (34) have described mutational alterations within the ATP binding site of TyrR that abolish repression by TyrR without interfering with its ability to activate. Amino acid switches at the analogous site within NtcA abolish activation while preserving the ability to repress. These distinctions suggest that TyrR may differ in mechanism from the σ^54-specific proteins of the NtcA superfamily.

We have identified and characterized a phosphatase activity intrinsic to TyrR. Our analysis of the system was facilitated by the fact that TyrR hydrolyzes standard phosphatase substrates. Zinc ion was shown to be important for the phosphatase activity of this prokaryotic regulatory protein. Using purified tryptic fragments, we localized the phosphatase activity within the 31-kDa fragment homologous to the central domain of the NtrC superfamily. Because the phosphatase activity was modulated by aromatic amino acids, a possible relationship between the regulatory function of TyrR and its phosphatase activity is suggested.

MATERIALS AND METHODS

Materials. p-Nitrophenyl phosphate, ATP, p-nitrophenol, and DEAE-Sepharose CL-6B were purchased from Sigma. ATP-γ-S was purchased from Boehringer Mannheim. Disodium 2-chloro-5-(4-methoxyspiro[1,2-dioxetane-3,2-(5’-chloro)-tricyclo[3.3.1.17-3]decane]-4-yl)-1-phenyl phosphate (CDP-Star)
phosphatase substrate and Sapphire-II were purchased from Tropix. Chelex-100 resin was from Bio-Rad. Phosphocellulose P11 was purchased from Whatman.

Stock solutions were made as follows: L-tyrosine, D-tyrosine, D-3-fluorotyrosine, L-phenylalanine, and L-phenylalanine were dissolved in 0.1 M HCl and adjusted to a pH of 8.0 with 2 M NaOH. Lysine was dissolved in buffer A (described in “Buffers” below). The concentration of each stock solution was 10 mM.

Strains and plasmids. pC100 contains the E. coli tyrR gene inserted into the NdeI and BamHI sites of pET3a (28). CHE6 was prepared by the PDS method as previously described (9); lysates with titers higher than 10^10 were retained for further use.

E. coli SP1566 is a derivative of BL21 with a kanamycin minicasette inserted in the tyrR gene, selected following transposon mutagenesis by A1105 (30) on the basis of resistance to 3-fluorotyrosine. Analogue resistance was reversed by the introduction of pC100.

Buffers. Tris buffer was 100 mM Tris (pH 8.0), 1 mM EDTA, 0.01% NaN3, 7 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Buffer A was 10 mM K2HPO4/KH2PO4 (pH 6.6) with 100 mM NaCl, 1 mM EDTA, 0.01% NaN3, 7 mM mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride. Phosphatase buffer was 100 mM HEPES (pH 6.5) with 2 mM ZnSO4, 10 mM MgSO4, and 100 mM NaCl. CDP-Star assay buffer contained 0.1 M diethanolamine (pH 8.0), 1 mM MgSO4, 2 mM ZnSO4, and 1/10 (vol/vol) Sapphire-II (luminescence enhancer). This buffer was stored at 4°C. Metal-free phosphatase buffer (buffer B) was made by adding 10 g of Chelex-100 resin to 500 ml of phosphatase buffer without ZnSO4 and MgSO4. After stirring for 4 h at room temperature, the resin was removed by centrifugation.

Purification of TyrR and removal of metals from TyrR preparations. The wild-type and mutant forms of the TyrR protein of E. coli were purified by a modification of the procedure of Cai and Somerville (9). To overexpress the wild-type and mutant TyrR proteins, plasmid-bearing derivatives of strain SP1566 (pC100) were induced by infection with CHE6, as described previously. Purification was carried out as follows.

(i) Purification. Cell pellet (10 g) was resuspended in Tris buffer (1 g of cell paste per 10 ml of buffer). Cells were broken in a French press (three passages at 2,000 lb/in2). The suspension was spun at 15,000 rpm (Beckman J21C) at 4°C for 60 min. The supernatant (95 ml) was loaded onto a 40-ml column (2.5 by 10 cm) of DEAE-Sepharose CL-6B. The column was washed with Tris buffer (about 300 ml) and then developed with a linear gradient of NaCl (0 to 0.5 M in a 500-ml volume). Fractions containing the TyrR protein (identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) were pooled. At this stage, the TyrR protein was 75 to 80% pure.

(ii) Step 2. Protein within the pooled column fractions (30 ml) was precipitated by adding solid ammonium sulfate to 75% saturation. After 2 h at 4°C, the precipitate was dissolved in buffer A (10 ml) and dialyzed overnight against 1 liter of the same buffer.

(iii) Step 3. The ammonium sulfate fraction was loaded onto a phosphocellulose P11 column (15 by 2.5 cm). The column was washed with 300 ml of buffer A. The column was developed with a linear gradient of NaCl from 0 to 0.5 M in a 500-ml column. The column was washed with Tris buffer (about 300 ml) and then developed with a linear gradient of NaCl (0 to 0.5 M in a 500-ml volume). Fractions containing the TyrR protein (identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) were pooled. At this stage, the TyrR protein was 75 to 80% pure.

(iv) Step 4. To remove divalent cations from the TyrR protein, the dialysis bag was transferred from buffer A to buffer B (with 20% glycerol). Chelex-100 resin (10 g) was added to buffer B. After dialysis for 24 h, the protein was aliquoted and stored at −20°C. The TyrR protein was at least 99% pure by SDS-PAGE analysis.

The TyrR protein of Haemophilus influenzae was purified as previously described (36).

Determination of TyrR concentration. TyrR protein concentrations were determined spectrophotometrically by using the extinction coefficient of Wilson et al. (33) for the E. coli protein or that of Zhu et al. (36) for the H. influenzae protein. Protein concentrations were also determined by a dye assay method (protein assay: Bio-Rad), using bovine serum albumin as the standard.

Limited trypsin digestion and purification of fragments. Limited trypsin digestion was carried out as previously described (9). The major digestion products, of 31 and 22 kDa, were purified chromatographically on polyethyleneimine-cellulose and stored at −20°C.

Autokinase activity and autophosphatase measurements. TyrR protein (1 mg/ml, 20 μl) was added to 180 μl of phosphatase buffer (5 mg of bovine serum albumin per ml was included as the carrier protein). [γ-32P]ATP (2 μl, 1 μM) was added, and the reaction mixture was incubated at 37°C. Samples (20 μl) were removed at various times after the addition of ATP. Each sample was immediately mixed with 100 μl of 10% trichloroacetic acid. The resulting protein precipitate was washed three times on a glass microfiter filter (Whatman) with 10 ml of 10% trichloroacetic acid and subjected to scintillation counting. In a parallel experiment, 20 μl of 2 mM nonradioactive ATP was added to a reaction mixture 20 s after the radioactive ATP. A sample was removed immediately after the nonradioactive ATP was added. Additional samples were taken at various time points thereafter.

Phosphatase assays. (i) Method 1. Substrate solution was p-nitrophenol phosphatase (9.5 mM) dissolved in phosphatase buffer. Each assay tube contained phosphatase buffer (100 μl), p-nitrophenyl phosphate solution (100 μl), and TyrR (10 μl). After incubation at 37°C for 3 h, reactions were stopped by adding 1 M Na2CO3 (1 ml) to each reaction mixture. The amount of product formed was calculated from the absorbance at 410 nm, with reference to a standard curve prepared using pure p-nitrophenol.

(ii) Method 2. CDP-Star assay buffer (400 μl), TyrR protein (4 μl), and CDP-Star substrate (7 μl) were mixed at room temperature. The chemiluminescence reached steady state after 30 min of incubation. The luminescence signal was measured on a luminescence meter (Monolitont 2010; Analytical Luminescence Laboratory) after 45 min of incubation.

RESULTS

TyrR proteins purified from E. coli and H. influenzae have phosphatase activity. The central domain (amino acids 206 to 425) of E. coli TyrR has substantial similarity to NtrC, which has ATPase activity (16, 22, 32). The TyrR protein of E. coli has both ATPase activity (10) and autokinase activity (Fig. 1). Phospho-TyrR, in the absence of any other protein, was readily dephosphorylated, as demonstrated by the rapid decline in acid-insoluble radioactivity when excess nonradioactive ATP was added after brief incubation of TyrR with [γ-32P]ATP. Suspecting that the lability of the protein-bound phosphoryl group reflected the activity of a phosphatase catalytic center, a study of this point was conducted. Using p-nitrophenol phosphatase as the substrate, it was found that the TyrR protein had low levels of phosphatase activity when assayed in buffer A containing 10 mM Mg2+ (see below). In further studies, this activity was found to be greatly enhanced by Zn2+ (see below). In the presence of excess Zn2+, 0.3 μmol of p-nitrophenyl phosphate hydrolyzed per min per μmol of TyrR protein was hydrolyzed. The rate of hydrolysis was directly proportional to the concentration, over a range of protein concentrations up to 20 μM (data not shown).

To rule out the possibility that the phosphatase activity of TyrR was attributable to trace contamination by an unrelated protein, a series of column fractions collected during the final step of purification (phosphocellulose P11) were assayed. Both
E. coli TyrR and the structurally related H. influenzae TyrR were examined. If the phosphatase were an intrinsic activity of TyrR, the activity in each column fraction should have been directly proportional to the protein concentration. The purity of each fraction was estimated by SDS-PAGE. Only protein bands (58 kDa for E. coli TyrR and 36 kDa for H. influenzae TyrR) corresponding to TyrR were detectable, indicating that each TyrR protein was homogeneous. For both E. coli TyrR and H. influenzae TyrR, the phosphatase activity peak aligned precisely with the protein peak (Fig. 2). In a further study, the two TyrR proteins were subjected to gel filtration chromatography on a column of Ultragel AcA34 (LKB) (1 by 120 cm). In both cases the protein peaks and the phosphatase activity peaks coincided exactly (data not shown). These results strongly support the conclusion that TyrR has intrinsic phosphatase activity.

**Stimulation of the phosphatase activity of TyrR by zinc.** Divalent cations often play key roles in the activities of phosphatases, either as structural elements or directly in catalysis. To explore whether specific divalent cations affect the phosphatase activity of TyrR, several different metal ions were added to the assay system. It was found that Zn\(^{2+}\) greatly stimulated the phosphatase activity of TyrR (over 57-fold). The stimulatory effect of other cations was mild (two- to eight-fold) (Table 1). Further studies showed that the phosphatase activity of E. coli TyrR was half-maximal at a concentration of about 250 \(\mu\)M and was slightly inhibited at Zn\(^{2+}\) concentrations above 4 mM (Fig. 3).

**Effects of trypsin digestion on phosphatase activity of TyrR.** Upon treatment with trypsin under standardized conditions, TyrR is converted to two discrete, stable fragments (9). The C-terminus (residues 468 to 513) is completely digested within the first few minutes, whereas a 22-kDa domain (residues 1 to 190) is very stable. The initially formed central domain of TyrR

![Graph showing coincidence of phosphatase activities and TyrR protein peaks during phosphocellulose P11 column chromatography.](image)

**FIG. 2.** Coincidence of phosphatase activities and TyrR protein peaks during phosphocellulose P11 column chromatography. (A) TyrR protein of E. coli. Chromatography was carried out as described in Materials and Methods. Assays were conducted at 37°C in the presence of p-nitrophenyl phosphate (5 mM), Zn\(^{2+}\) (2 mM) and ATP-\(\gamma\)-S (1 mM). A 5-\(\mu\)l sample of each fraction was assayed. The TyrR concentration is shown as the \(A_{280}\) and the amount of p-nitrophenol produced is shown as the \(A_{410}\), phosphatase activity (in \(A_{410}/2.5 \text{ h/5-\mu l sample}\)); \(\diamond\), absorbance at 280 nm. (B) TyrR protein of H. influenzae. Assays were carried out under the same conditions as those used for the E. coli TyrR protein. \(\circ\), absorbance at 280 nm; \(\Box\), phosphatase activity (in \(A_{410}/2.5 \text{ h/5-\mu l sample}\)).

![Graph showing effect of high concentrations of Zn\(^{2+}\) on the phosphatase activity of TyrR.](image)

**FIG. 3.** Effect of high concentrations of Zn\(^{2+}\) on the phosphatase activity of TyrR. A series of solutions were made by dissolving ZnSO\(_4\) in Zn\(^{2+}\)-free phosphatase buffer. Assays were carried out at 37°C in the presence of p-nitrophenyl phosphate (5 mM), ATP-\(\gamma\)-S (1 mM), and TyrR (10 \(\mu\)M). Assays were terminated, and absorbancies were measured as described in Materials and Methods.

<table>
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<th>Metal ion</th>
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</tr>
<tr>
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</tr>
<tr>
<td>Co(^{2+})</td>
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*The buffer was treated with Chelex-100 resin to remove traces of metal ions. Other procedures are described in Materials and Methods. The cations were added as chloride salts at final concentrations of 2 mM. The activity of the buffer-only assay (0.007 mol of p-nitrophenyl phosphate/min/mol of TyrR) was set at 1.0.*
(residues 191 to 467) slowly undergoes further cleavage upon prolonged exposure to trypsin (9). To gain insight into the location of the phosphatase activity of TyrR, a kinetic experiment was carried out. Samples taken during the course of a limited trypsin digestion were assayed for phosphatase activity. There was an initial increase in activity, followed by a steady decrease (data not shown). Phosphatase activity remained detectable over time but continuously decreased for 30 min without reaching a stable plateau value. This result suggests that the phosphatase activity was associated with neither the 22-kDa N-terminal domain nor the C terminus of TyrR. In the initial phase of trypsin digestion, fragments of 31 kDa are abundant. At later times, the 31-kDa fragment undergoes further digestion into smaller fragments (9). The survival kinetics of phosphatase activity during trypsin digestion thus suggested that the phosphatase activity was most likely associated with the 31-kDa tryptic domain of TyrR.

**Purified 31-kDa tryptic fragment of TyrR retains phosphatase activity.** The TyrR protein of *H. influenzae* shows a high degree of homology to the second domain and C-terminal segment of *E. coli* TyrR (36). The fact that *H. influenzae* TyrR lacks an N-terminal domain analogous to that found within *E. coli* TyrR, while exhibiting phosphatase activity (see below), suggested that the phosphatase catalytic site of *E. coli* TyrR does not lie within the amino-terminal half. By chromatography it is possible to separate the two main tryptic fragments of TyrR at a purity of over 90%. When p-nitrophenyl phosphate was used as substrate, neither fragment had detectable phosphatase activity. However, assays of the 31-kDa fragment using CDP-Star, a phosphatase substrate that releases a chemiluminescent product, yielded positive results. The chromatographically purified 22-kDa fragment had only 6.9% of the phosphatase activity of the larger fragment (Fig. 4). This low activity may reflect slight contamination by the 31-kDa fragment. Both the intact *E. coli* TyrR protein and the *H. influenzae* TyrR protein could also hydrolyze CDP-Star. The specific phosphatase activity of the intact *E. coli* TyrR protein was about 50% of that of the 31-kDa fragment, under identical conditions (data not shown). Thus, the most likely location for the phosphatase catalytic center is within the segment bounded by residues 191 and 467.

A possible explanation for the inactivity of the 31-kDa fragment toward *p*-nitrophenyl phosphate is that this compound is structurally similar to aromatic amino acids. The phosphatase activity of the 31-kDa fragment was more susceptible to inhibition by tyrosine and its analogues than was that of intact TyrR (see below), suggesting that the N terminus of TyrR may be masking a tyrosine binding site. Viewed as a tyrosine analogue, *p*-nitrophenyl phosphate could function either as a substrate or as an inhibitor of phosphatase activity. It is also possible that intact TyrR and its 31-kDa fragment may have different substrate specificities. In fact, *p*-nitrophenyl phosphate did inhibit the phosphatase activity, measured by chemiluminescence, of the 31-kDa fragment (Fig. 5). At 5 mM *p*-nitrophenyl phosphate, inhibition was more than 60% but not complete. This result may indicate that some combination of both the effects discussed above accounts for the inability of the 31-kDa fragment to hydrolyze *p*-nitrophenyl phosphate. Alternatively, *p*-nitrophenyl phosphate and CDP-Star could bind to different sites.

**ATP affects phosphatase activity of TyrR.** Computer analysis suggests that TyrR has a Walker type A nucleotide binding site within the central domain, which is consistent with a range of observations demonstrating ATP binding (2, 3) and hydrolysis (10; F. Ortega and R. L. Somerville, unpublished results). To investigate the effect of ATP on the phosphatase activity of TyrR, ATP-γ-S was employed. The inability of TyrR to hydrolyze ATP-γ-S has been documented (33). This compound weakly inhibited the phosphatase activity of full-length TyrR and strongly inhibited the phosphatase activity of the 31-kDa tryptic fragment. For intact TyrR, inhibition was half-maximal...
was therefore routinely included in phosphatase assay reaction mixtures in all subsequent experiments for two reasons: (i) ATP-γ-S is required in order to demonstrate an effect of tyrosine and its analogues, and (ii) the half-maximal inhibitory concentration of ATP-γ-S is approximately 1 mM.

1-Tyrosine and tyrosine analogs inhibit phosphatase activity of TyrR protein. 1-Tyrosine is an important TyrR ligand. In vitro, 1-tyrosine and tyrosine analogs are unable to bind to TyrR unless ATP is present (2). The binding of 1-tyrosine stimulates the ATPase activity of the TyrR protein (10; F. Ortega and R. L. Somerville, unpublished results), and affects the course of proteolytic digestion of TyrR by trypsin (9). A number of compounds structurally related to tyrosine were tested for their effect on phosphatase activity. In the absence of ATP, 1-tyrosine and its analogs had little effect (data not shown). When 1 mM ATP-γ-S was present, the phosphatase activity of the TyrR protein was inhibited by 1-tyrosine and several tyrosine analogues, but not by l-lysine. Half-maximal inhibition was observed at ligand concentrations of approximately 1.25 mM (Fig. 7A). Parallel series of experiments were carried out on the 31-kDa tryptic fragment of TyrR, using CDP-Star as a substrate. When ATP-γ-S was absent, none of the amino acids affected the phosphatase activity of the 31-kDa tryptic fragment (data not shown). When 1 mM ATP-γ-S was present, the phosphatase activity of the 31-kDa tryptic fragment was inhibited by each of the compounds except l-lysine. Half-maximal inhibition was observed at ligand concentrations of approximately 100 μM (Fig. 7B).

The concentrations at which tyrosine and its analogues exerted inhibitory effects on the phosphatase activity of TyrR or its 31-kDa subfragment were considerably higher than the concentration of tyrosine (14 to 22 μM) that yields half-maximal inhibition of the ATPase activity of native TyrR (F. Ortega and R. L. Somerville, unpublished results). The effect of tyrosine on the phosphatase activity probably does not directly reflect some modification by tyrosine of the ATPase catalytic center. However, this possibility must be tempered by the fact that Zn2+ is absent from standard ATPase assay mixtures and was present whenever the phosphatase activity was being measured.

Possible role for zinc in TyrR. To further investigate the role of Zn2+ in the TyrR system, we conducted spectral studies of TyrR in the presence and absence of Zn2+. Prior to analysis, the TyrR protein was dialyzed against buffer B containing Chelex-100 resin (see Materials and Methods). If Zn2+ is either a structural element of TyrR or involved in catalysis or both, this metal ion could induce conformational changes in TyrR which might be detectable spectroscopically. By conventional absorption spectroscopy, there were no observable changes in the TyrR protein in the presence or absence of Zn2+ over the wavelength range of 220 to 300 nm. In fluorescence spectroscopy, there was a small shift in emission wavelength (340 to 338 nm) after Zn2+ was added. However, the principal effect of Zn2+ was to decrease the emission intensity. In the presence of Zn2+, there was a drop of approximately 15% in emission intensity. As the concentration of the Zn2+ was increased, fluorescence decreased, in a saturable fashion. The loss of intensity was half-maximal at a concentration of Zn2+ of 200 μM. This concentration is similar to that which gave half-maximal stimulation of phosphatase activity (Fig. 3). At Zn2+ concentrations higher than 1 mM, there was little further decrease in the intensity of emission (Fig. 8). This result is consistent with either a structural or a catalytic role for Zn2+. Reciprocal effect of zinc on ATPase activity and phosphatase activity of TyrR. If Zn2+ plays a role in the action of TyrR, its
binding could affect the intrinsic ATPase activity of the protein. To test this possibility, Zn\(^{2+}\) was added at progressively increasing concentrations to the ATPase assay system, as previously described (10). Zn\(^{2+}\) was found to inhibit the ATPase activity of TyrR (Fig. 9). Half-maximal inhibition was observed at a concentration of about 100 \(\mu\)M. When Mg\(^{2+}\) was present at high concentrations (20 \(\text{mM}\)) in the assay system, Zn\(^{2+}\) remained potent, with the same half-maximal inhibitory concentration (data not shown). This result makes it unlikely that Zn\(^{2+}\) exerts its effect by competing with Mg\(^{2+}\), for example, by forming a complex with ATP, thereby lowering the effective concentration of the ATP-Mg complex. The reciprocal effect of Zn\(^{2+}\) on the phosphatase activity and ATPase activity of TyrR (Fig. 9) indicates that Zn\(^{2+}\) ion can influence the known catalytic centers of the TyrR protein.

**Phosphatase activity of mutationally altered TyrR proteins.** Among several purified mutant TyrR proteins that were examined, certain alterations in structure (S493A and H494N) did not affect phosphatase activity, while others resulted in either increased (L3K) or decreased (T495A, T495E, and T495D) activity. The third class (T495A, T495E, and T495D) of proteins contain alterations in the putative DNA-binding elements (Fig. 10). These observations strengthen the notion that phosphatase activity is an intrinsic property of TyrR that can be modulated by changes at both ends of the protein.

**Effects of phosphatase inhibitors.** Alkaline phosphatase is sensitive to 1 to 5 \(\text{mM}\) tetrathiole, while 5 to 10 \(\text{mM}\) tartrate is a known inhibitor of acid phosphatase activity. Both type 2A and type 2B serine/threonine phosphatases are sensitive to okadaic acid, while vanadate and tungstate, etc., can inhibit protein tyrosine phosphatase (20). For TyrR, none of the above compounds was able to inhibit phosphatase activity. Fluoride ion is widely used as a broad-spectrum phosphatase inhibitor. When NaF was tested, inhibition was observed, with a half-maximal inhibitory concentration of 25 \(\text{mM}\) (data not shown). This result suggests that TyrR is a novel phosphatase.

**Turnover number and pH dependence.** To determine the turnover number that governs the phosphatase activity of TyrR, reaction conditions were optimized and kinetic constants were determined using \(p\)-nitrophenyl phosphate as the substrate. Enzyme activity was also examined as a function of pH. There was no activity above a pH of 9.0; TyrR tends to precipitate below a pH of 5.5. The optimal pH for TyrR phosphatase activity was 6.5 (data not shown). The optimal Zn\(^{2+}\) concentration was 2 \(\text{mM}\). Under standard conditions (pH 6.5, 1 \(\text{mM}\) ATP-\(\gamma\)-S, 2 \(\text{mM}\) Zn\(^{2+}\)), 0.37 mol of \(p\)-nitrophenyl phosphate/min/mol of TyrR protein monomer was hydrolyzed at 37°C.

**DISCUSSION**

Although it has been conclusively demonstrated that the TyrR protein has phosphatase activity, the natural substrate remains unknown. The phosphatase activity of phosphorylated TyrR (Fig. 1) could be either intramolecular or intermolecular in nature. It is likewise unclear whether or how the phosphatase activity of TyrR might affect the function of this protein in regulating transcription. The phosphatase activity of TyrR protein is inhibited by \(\tau\)-tyrosine and its analogues and to a lesser extent by ATP, raising the possibility that the TyrR protein executes its transcriptional regulatory function by a mechanism that involves phosphatase activity.

In solution, TyrR binds ATP (3). Two putative ATP-binding sites (residues 234 to 240 and 291 to 297) were found by computer analysis, with the first binding site an adenylate kinase-type ATP-binding site. In studies of the ATPase activity of TyrR (F. Ortega and R. L. Somerville, unpublished results), two nonidentical ATP binding sites were inferred. A high-affinity site binds ATP with a \(K_a\) of approximately 1.2 \(\mu\)M; a second low affinity site binds ATP with a \(K_a\) of 900 \(\mu\)M. Subsequent to interaction with ATP, TyrR can bind tyrosine or...
The binding of tyrosine can stimulate ATP hydrolysis threefold (F. Ortega and R. L. Somerville, unpublished results). ATPase activity is believed to be required for the activation function of the NtrC family of proteins (4, 20). Although no direct relationship has been established between transcriptional control by TyrR and its ATPase activity, mutations within or near ATP binding sites (G237, E274, G285, and E302) disable the repression function without altering the transcriptional activation capability (1, 23, 24, 34). TyrR undergoes autophosphorylation in a manner that depends on the concentration of ATP (Fig. 1). Phospho-TyrR protein was readily dephosphorylated, leading us to postulate that the TyrR possessed phosphatase activity.

None of the standard phosphatase inhibitors except NaF affected the phosphatase activity of TyrR. The effect of fluoride ion and the half-maximal inhibitory concentrations of fluoride ion were basically identical with and without zinc ion. This suggests that fluoride ion does not inhibit the phosphatase activity of TyrR by sequestering the Zn\(^{2+}\). On the other hand, the solubility of ZnF\(_2\) is about 150 mM (for F, the solubility is 300 mM), which is far above the half-maximal inhibitory concentration of NaF. This also suggests that fluoride ion does not act by affecting the availability of Zn\(^{2+}\). Inhibition appears to be caused directly by fluoride ion. The effects of this inhibitor further support the existence of intrinsic phosphatase activity within TyrR and make it unlikely that our enzyme preparations were contaminated with a different phosphatase.

Amino acid switches near the extremities of the TyrR protein were found to alter its phosphatase activity (Fig. 10). The 31-kDa tryptic fragment of TyrR had about twice as much phosphatase activity as the intact protein when CDP-Star was used as the substrate. This result was consistent with kinetic
metal ion could function as a structural element, as an inhibitor of the catalytic site, or both. As a structural element, metal ion could function as a structural element, as an inhibitor of the catalytic site, or both. As a structural element, metal ion could function as a structural element, as an inhibitor of the catalytic site, or both. As a structural element, metal ion could function as a structural element, as an inhibitor of the catalytic site, or both.

The discovery that Zn\textsuperscript{2+} affects in reciprocal fashion the phosphatase activity of TyrR naturally acts on effector proteins other than itself, the N terminus of TyrR could sterically inhibit the phosphatase activity of the second domain by preventing access by the substrate to the catalytic site. Proteolytic removal of the N-terminal domain would make the active site more accessible, thus increasing the phosphatase activity. If the natural substrate for the phosphatase activity of TyrR lies within its own N-terminal domain, the activity we have described could drastically underrepresent the true phosphatase activity of TyrR. Removing or altering the conformation of the N terminus, as in the L3K mutant, could also increase the ability of standard substrates (e.g., p-nitrophenyl phosphate and CDP-Star) to reach the active site, thereby increasing the observed phosphatase activity.

Although no previous reports have suggested that zinc affects the function of prokaryotic transcriptional factors, zinc ions are key structural components of many proteins (17). The finding that Zn\textsuperscript{2+} affects in reciprocal fashion the phosphatase activity and the ATPase activity of TyrR (Fig. 9) raises the possibility that Zn\textsuperscript{2+} plays a role in modulating the expression of the genes of the TyrR regulon.

There are no cysteine- or histidine-rich clusters within the TyrR protein that resemble those found in many eukaryotic regulatory proteins. This tends to suggest that Zn\textsuperscript{2+} binds to TyrR in a distinct way, as, for example, in serine/threonine phosphatase 1, where widely separated Asp, Asn, and His residues are responsible for metal ion binding (6). The fact that zinc can greatly stimulate the phosphatase activity of TyrR suggests two possibilities. First, TyrR may be a metalloprotein, having Zn\textsuperscript{2+} as an essential structural component. The bound metal ion could function as a structural element, as an ingredient of the catalytic site, or both. As a structural element, Zn\textsuperscript{2+} could hold TyrR protein in a particular conformation by chelating to certain amino acid residues, or it could affect the structure of TyrR dimers by forming a bridge between two TyrR monomers. Second, as part of the catalytic center, Zn\textsuperscript{2+} could stabilize the transition state or play a role in electron relay during the catalytic process, as it does in carboxypeptidase (11) and in serine/threonine phosphatase 1 (6).

TyrR has many properties in common with carboxypeptidase. First, zinc ions are required for the enzymatic activities of both proteins; second, excess Zn\textsuperscript{2+} inhibits both the enzymatic activity of carboxypeptidase and the phosphatase activity of TyrR (25); third, upon adding zinc ion to metal-free apoenzymes, both proteins showed a decrease in fluorescence intensity and a slight change in emission wavelength (14) (Fig. 8); and fourth, both proteins lack cysteine- or histidine-rich regions as identifiable zinc-binding motifs. These similarities suggest analogous mechanisms for the binding of zinc. Possibly zinc has a functional role in TyrR similar to that in carboxypeptidase. Since X-ray crystallography showed that a tyrosine residue can bind to the hydrophobic pocket of carboxypeptidase so that its carbonyl group interacts with bound Zn\textsuperscript{2+} (11), it is not unreasonable to imagine that TyrR could have a Zn\textsuperscript{2+} binding environment and a hydrophobic pocket similar to that of carboxypeptidase. The phosphatase activity of TyrR might be regulated via the binding or release of tyrosine or phenylalanine. Regulation of phosphatase activity could in turn affect the DNA-binding function of TyrR, for example, by changing its phosphorylation state, thus altering the ability of the N terminus to interact with the RNA polymerase or other effectors.

Under optimal conditions, the turnover number of TyrR was 0.37 mol of p-nitrophenyl phosphate/min/mol of protein at 37°C, which is essentially identical to that observed for the ATPase activity of TyrR (0.4 mol of ATP/min/mol of protein) (10). Although the similarities in turnover number could be coincidental, this fact forces one to consider a possible connection between the phosphatase and the ATPase activities of the TyrR protein. The reciprocal effect of zinc ion on the ATPase activity and phosphatase activity of TyrR (Fig. 9) provides evidence for a functional connection between the two catalytic centers. In addition, tyrosine and its analogues not only stimulate the ATPase activity of TyrR (1, 10) but also inhibit the phosphatase activity of TyrR (Fig. 7). If further studies can clarify the connection between the ATPase activity...
and the phosphatase activity of TyrR, our understanding of how TyrR mediates transcriptional regulation would be improved.

ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Public Health Service (GM22311) and the U.S. Army Research Office (DAAH 49-95-1-0138).

REFERENCES


