Altered Oligomerization Properties of N316 Mutants of *Escherichia coli* TyrR

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The transcriptional regulator TyrR is known to undergo a dimer-to-hexamer conformational change in response to aromatic amino acids, through which it controls gene expression. In this study, we identified N316D as the second-site suppressor of *Escherichia coli* TyrR<sup>E274Q</sup>, a mutant protein deficient in hexamer formation. N316 variants exhibited altered in vivo regulatory properties, and the most drastic changes were observed for TyrR<sup>N316D</sup> and TyrR<sup>N316R</sup> mutants. Gel filtration analyses revealed that the ligand-mediated oligomer formation was enhanced and diminished for TyrR<sup>N316D</sup> and TyrR<sup>N316R</sup>, respectively, compared with the wild-type TyrR. ADP was substituted for ATP in the oligomer formation of TyrR<sup>N316D</sup>.

TyrR is a transcriptional regulator of genes mainly involved in the metabolism of aromatic amino acids in bacteria. Of the known TyrR proteins, *Escherichia coli* TyrR is the most extensively studied and is known to regulate expression of at least eight genes responsible for biosynthesis and transport of aromatic amino acids (1, 4, 5, 11, 12, 16, 18, 19, 22, 23, 25–29) (Fig. 1A). In addition, it has recently been shown that the folA gene, encoding dihydrololate reductase, is also regulated by TyrR (29). The protein comprises three domains with different functions (Fig. 1B). The N-terminal domain has a site for aromatic amino acid binding (23) and a region that interacts with the α-subunit of RNA polymerase to stimulate transcription initiation (12). The TyrR homolog of *Haemophilus influenzae* lacks this domain, and therefore it is incapable of transcriptional activation (9, 10, 24, 30, 31). The central domain shows a high sequence similarity to those of σ<sup>54</sup>-dependent enhancer-binding proteins (NtrC family), but TyrR differs from them in that it regulates transcription from σ<sup>70</sup>-dependent promoters to reflect the lack of the GAFTGA motif that is essential for contact with the σ<sup>54</sup> subunit. The NtrC family, including TyrR, belongs to the AAA + (ATPases associated with diverse cellular activities) superfamily (4, 15, 22). A common feature of the AAA + superfamily is the formation of a ring-shaped oligomer in response to environmental stimuli (15). TyrR exists as a dimer in solution, but in the presence of ATP and tyrosine (or a high concentration of phenylalanine) it changes its conformation from a dimer to a hexamer (1, 11, 25, 26). This central domain-dependent oligomerization is triggered by the binding of aromatic amino acids to another binding site located in this domain (an ATP-dependent site) (25). The C-terminal domain has a helix-turn-helix motif, which is structurally similar to that of the cyclic AMP receptor protein (3), and binds to DNA with a consensus sequence of TGTAAN<sub>5</sub>TTTACA (TyrR box) (Fig. 1A) (19).

The promoter-operator regions of the TyrR regulon encompass one to three TyrR boxes with different affinities, as summarized by Pittard et al. (18) (Fig. 1A). A box to which a TyrR dimer can bind in the absence of an aromatic amino acid cofactor usually has high sequence identity with the consensus and is called a strong box. On the other hand, a site having low sequence identity is called a weak box. TyrR binds to the weak box only when a strong box is juxtaposed nearby on the same face of the helix and when the TyrR dimer bound to the strong box forms an oligomer in the presence of cofactors (cooperative binding). TyrR regulates transcription either positively or negatively by changing its dimer-hexamer conformation and by binding to the strong/weak boxes (Fig. 1A).

In a previous study, Kwok et al. found that the substitution of glutamine for glutamic acid at position 274 (E274Q) of *E. coli* TyrR renders the protein deficient in oligomer formation (11). This mutant TyrR exhibited normal binding to ATP, but the tyrosine-mediated hexamer formation was severely impaired, which suggested an important role for this residue in the process of dimer-hexamer conversion. In the present study, we tried to isolate second-site suppressors of this mutant protein and identified asparagine-316 as a critical residue in the fine-tuning of the oligomeric state of TyrR.

**Mutations that suppress the inability of *Erwinia herbicola* TyrR<sup>E275Q</sup> to activate the tpl promoter.** We have previously studied the TyrR protein of *Erwinia herbicola* (6, 8, 21). E274 of *E. coli* TyrR corresponds to E275 of *E. herbicola*, and the corresponding mutant (TyrR<sup>E275Q</sup>) showed an impaired ability to activate tpl (Table 1), possibly due to a deficiency in hexamer formation (the action mechanism of TyrR on the tpl promoter is described later), and was targeted for isolation of a second-site suppressor by introducing random mutations.

A mutant tyrR<sup>E275Q</sup> of *E. herbicola* library was screened for a recovered ability of its product to activate the Φ<sup>tpl</sup>′-lac gene (pTK815) (6). Error-prone PCR and subsequent construction of the plasmid library was performed as described previously (6) except that pTK815 (p15A replicon bla<sup>+</sup> tyrR<sup>E275Q</sup> *E. herbicola*)
was used as the template for PCR. The plasmid library was used to transform *E. coli* Δ*tyrR* strain TK596 [F− ara Δ(lac-pro) thi Δ*tyrR*-kan + Δ(ogl-recA)306::Tn10] (6) carrying pTK871. Transformants were visually screened for enhanced formation of blue color on LB plates (14) containing 1 mM 5-bromo-4-chloro-3-indolyl-D-galactopyranoside, and four transformants were selected for further analysis. Sequence analysis of the four mutant *tyrR* genes revealed that all alleles still contained the E275Q substitution; nevertheless, the abilities of their products to activate *tpl* were recovered to a level comparable to that of wild-type TyrRE. *coli* K-12 MG1655; *tpl*, tyrosine phenol lyase of *E. herbicola*.

![FIG. 1](image-url)

**A** The promoter-operator regions of the TyrR regulon. TyrR binding sites are represented by black (strong boxes) and white (weak boxes) rectangles. Transcription initiation sites (+1) and the −35 and −10 promoter regions are indicated. Regulatory modes of TyrR on the respective promoters in the presence of tyrosine (Tyr) and phenylalanine (Phe) are indicated as R (repression) or A (activation). (B) Domain structure of TyrR<sub>E. coli</sub>. The numbering starts at the initiation codon. Roles of the respective domains are indicated. The ATP-binding Walker A and B motifs and the helix-turn-helix DNA-binding motif (HTH) are shown by black and shaded boxes, respectively, and the glutamic acid-274 (E274) and asparagine-316 (N316) residues are indicated.

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coli TyrR instead of *E. herbicola* TyrR for further analysis because the corresponding *E. coli* TyrR mutant (TyrR<sup>N316D</sup>) did not cause such severe growth retardation. Site-directed mutagenesis of the *tyrR* gene was carried out by the QuikChange method (Stratagene) using pTK723 (p15A replicon *bla*<sup>+</sup> *tyrR*<sup>E. coli</sup>) (6) as a template and oligonucleotides with the desired mutations. The entire fragment used for later manipulation was sequenced to ensure that no base changes other than those planned had occurred.

Three genes (*aroF*, *tyrP*, and *tpl*) of the TyrR regulon were transcriptionally fused to *lac* genes (6) and used as reporters. The *aroF* and *tyrP* genes of *E. coli* encode tyrosine-repressible 3-deoxyarabinoheptulosonate 7-phosphate synthase and tyrosine-specific permease, respectively (17–19), and the *tpl* gene of *E. herbicola* encodes tyrosine phenol lyase (6–8). In the cases of *aroF* and *tyrP*, a strong box(es) is located upstream of, and a weak box overlaps with, the −35 promoter (Fig. 1A). When the ligand-mediated cooperative binding of TyrR occurs in this region, RNA polymerase is eliminated from the promoter, which results in repression of expression (26). In the presence of tyrosine, TyrR represses the expression of *aroF* and *tyrP*, while in the presence of phenylalanine TyrR represses *aroF* but activates *tyrP* (26, 28). Phenylalanine-mediated activation of *tyrP* occurs when the ligand binds to the N-terminal ATP-independent site of the TyrR dimer bound to the strong box (23). Whereas hexamer formation of TyrR causes repression of *aroF* and *tyrP*, the *tpl* promoter is activated by ligand-mediated hexamerization of TyrR bound to the three distant boxes (Fig. 1A) (2, 8, 20). Activation of *tpl* also requires the binding of the aromatic amino acid to the N-terminal ATP-independent site.

A plasmid carrying one of three reporter genes [Φ(*aroF<sup>−</sup>-lac*) (pTK588), Φ(*tyrP<sup>−</sup>-lac*) (pTK589), or Φ(*tpl<sup>−</sup>-lac*) (pTK871)] (6) was introduced into the *E. coli* strains with the mutant *tyrR* genes on a compatible plasmid (Fig. 2 legend). These strains were grown in M63−0.2% (wt/vol) glucose minimal medium (14) supplemented with 1 μg/ml thiamine-HCl and 30 μg/ml proline in the absence and presence of 1 mM phenylalanine or tyrosine. The amount of each TyrR<sup>N316</sup> variant in the cells was essentially the same, as revealed by immunoblotting using an anti-TyrR antibody (data not shown).

N316 substitutions had varied effects on the regulatory properties of TyrR, and among the mutants, significant changes were observed for TyrR with N316D/E and N316R/K substitutions (Fig. 2). The basal expression levels of Φ(*aroF<sup>−</sup>-lac*) and Φ(*tyrP<sup>−</sup>-lac*) in the cells carrying TyrR<sup>N316D</sup> *E. coli* were significantly lower than those in the cells carrying wild-type TyrR (Fig. 2A and B). In the presence of phenylalanine, TyrR<sup>N316D</sup> *E. coli* slightly activated *aroF* expression, unlike TyrR<sup>WT</sup> *E. coli*, and stimulated *tyrP* transcription, similar to TyrR<sup>WT</sup> *E. coli*. In both cases, the expression levels were considerably lower than those for the cells carrying wild-type TyrR (Fig. 2A and B). The ratio of phenylalanine-mediated activation of *tpl* by TyrR<sup>N316D</sup> *E. coli* was 2.5-fold higher than that caused by TyrR<sup>WT</sup> *E. coli* (Fig. 2C). The *aroF* and *tyrP* expression levels in the cells carrying TyrR<sup>N316D</sup> *E. coli* dropped to a basal level in the presence of tyrosine, and the expression levels were almost equal to those observed for the cells carrying wild-type TyrR grown in the presence of tyrosine. Activation of *tpl* in the presence of tyrosine was enhanced in the cells carrying TyrR<sup>N316D</sup> *E. coli* compared with the cells carrying wild-type TyrR. These results, i.e., the significant decrease of *aroF* and *tyrP* expression levels and increase in *tpl* expression, suggested an enhanced ability of this mutant TyrR to self-associate. In any case, the N316D substitution overcame the effects caused by the E274Q substitution (E274Q versus N316D E274Q), indicating that N316D is a second-site suppressor mutation of E274Q. In the strain carrying TyrR<sup>N316E</sup> *E. coli*, a similar regulatory mode was observed for *aroF*, *tyrP*, and *tpl* expression levels (Fig. 2A, B, and C). It seems that replacement of N316 with acidic residues promotes self-association of the TyrR protein.

By contrast, the basal expression levels of *aroF* and *tyrP* were derepressed in the strains carrying TyrR<sup>N316K</sup> *E. coli* of TyrR<sup>N316R</sup> *E. coli* compared with the strains with wild-type TyrR, and the expression levels were almost equal to those of the *tyrR*-null mutants (Fig. 2A and B). In the cells carrying TyrR<sup>N316K/R</sup> *E. coli*, no repressive effect was observed for *aroF* expression in the presence of phenylalanine, but significant repression was observed in the presence of tyrosine (Fig. 2A). The ratio of activation of *tyrP* in the presence of phenylalanine was enhanced in the cells carrying N316K/R mutants compared with that in the cells with wild-type TyrR, but the ratio of repression in the presence of tyrosine was slightly decreased (Fig. 2B). The basal expression of *tpl* did not change between the cells carrying wild-type and N316K/R mutant TyrR proteins, but the ligand-mediated activation was considerably lower in the cells carrying TyrR<sup>N316K/R</sup> *E. coli* compared with the cells carrying TyrR<sup>WT</sup> *E. coli* (Fig. 2C). These results, even though tyrosine-mediated repression of *aroF* and *tyrP* was still observable, suggested a diminished ability of the mutant TyrR proteins to form an oligomer.

TyrR<sup>N316L</sup> also derepressed *aroF* and *tyrP* expression levels to a lesser extent but did not affect *tpl* expression (Fig. 2). Replacement of N316 with alanine, cysteine, or histidine did

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### Table 1. Amino acid substitutions that suppress the impaired ability of *E. herbicola* TyrR<sup>E275Q</sup> to activate *tpl*

<table>
<thead>
<tr>
<th>Allele present on pACYC177&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sp act of β-galactosidase (Miller units)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Mutation(s) (putative amino acid replacement(s))&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>24 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>tyrR&lt;sup&gt;e&lt;/sup&gt;</td>
<td>160 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>tyrR&lt;sup&gt;E275Q&lt;/sup&gt;</td>
<td>55 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>tyrR7</td>
<td>160 ± 0.31</td>
<td>AAC for N-324 to GAC for D (N324D)</td>
</tr>
<tr>
<td>tyrR8</td>
<td>120 ± 3.4</td>
<td>CGG for R-215 to CAG for Q (R215Q);</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAC for N-324 to GAC for D (N324D)</td>
</tr>
<tr>
<td>tyrR9</td>
<td>210 ± 1.0</td>
<td>GCC for A503 to ACC for T (A503T)</td>
</tr>
<tr>
<td>tyrR10</td>
<td>160 ± 4.5</td>
<td>AGT for S-25 to GGT for G (S25G);</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAC for N-324 to GAC for D (N324D);</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAA for E-390 to GAG for E (E390E)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The respective *tyrR*<sup>E. herbicola</sup> genes were placed under the control of the wild-type promoter of tyrR on the pACYC-derived plasmid pTK774 (6), and the resulting plasmids were introduced into the Δ(phil−lac)-carrying strain (pTK871/TK969).

<sup>b</sup> The strains were grown in LB medium at 37°C, and the assays were done with the method described by Miller (14). The values are means ± standard deviations.

<sup>c</sup> All mutant tyrR alleles possessed the E275Q substitution in addition to the newly introduced mutation(s) indicated.
not significantly alter the regulatory property of the E. coli TyrR protein (Fig. 2).

Oligomerization of E. coli TyrRWT, TyrRN316D, and TyrRN316R proteins. The TyrR\textsuperscript{N316D} \textit{E. coli} and TyrR\textsuperscript{N316R} \textit{E. coli} proteins showed significantly altered in vivo regulatory properties; therefore, we analyzed their oligomer formation abilities in the presence of the ligands using gel filtration chromatography, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3). To obtain the purified proteins, the respective \textit{tyrR} genes were placed under the control of a T7 promoter (pET-3a; Novagen), and the resulting plasmids were introduced into a \textit{F}\textsuperscript{−} \textit{hsdS}(\textit{rB} \textsuperscript{−} \textit{mB} \textsuperscript{−}) \textit{gal ompT} \textit{thi} \textit{ara} \textit{lac} \textit{gal} \textit{recA} \textit{kan}\textsuperscript{−}\textit{cat}\textsuperscript{−} \textit{DE3}\textsuperscript{−} \textit{lysogenized tyrR}-deficient derivative of BL21 [\textit{F}\textsuperscript{−} \textit{hsdS}(\textit{rB} \textsuperscript{−} \textit{mB} \textsuperscript{−}) \textit{gal ompT} \textit{tyrR}:\textit{kan}\textsuperscript{−}] (YG110). The purification process was essentially the same as described by Argaet et al. (1) except that Superdex 200 HR10/30 (GE Healthcare) was used instead of Superose 12. Size exclusion chromatography (Superdex 200 HR10/30 column) was carried out by injecting a protein solution (1 ml) containing 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 1 mM EDTA, 0.1 mM dithiothreitol, 25 \(\mu\)g/ml phenylmethylsulfonyl fluoride, 10 mM MgCl\textsubscript{2}, and purified TyrR (5 \(\mu\)M) with or without 100 \(\mu\)M ATP (or ADP) and the indicated concentra-
activation of tlp. To our surprise, TyrR<sub>N316D</sub><sub>E. coli</sub> shifted its size to a higher molecular weight in the presence of ADP and Phe or Tyr, whereas wild-type TyrR absolutely requires ATP for its oligomerization (4) (Fig. 3A versus B). Even in the presence of ADP alone, a slight shift in size was observed for TyrR<sub>N316D</sub><sub>E. coli</sub>, suggesting that ADP could serve, if only partially, as an alternative to ATP for this mutant protein in the oligomerization process. The conformational change of this mutant protein in the presence of aromatic amino acids was reversible (data not shown).

In contrast, TyrR<sub>N316R</sub><sub>E. coli</sub> was eluted at a position corresponding to a dimer under all conditions tested, though a slight shift in size was seen when the tyrosine concentration was raised to 750 μM (Fig. 3C). These properties also explain the altered regulatory mode of this protein in vivo, i.e., derepression of aroF and tyrP and decreased activation of tlp.

**Concluding remarks.** N316 is located just downstream of the Walker B motif in the central domain of <em>E. coli</em> TyrR and is conserved in all the TyrR proteins isolated so far. In this study, we demonstrated that the N316 substitution had varied effects on the in vivo regulatory properties of TyrR, and we also showed the altered in vitro oligomerization properties of TyrR<sub>N316D</sub> and TyrR<sub>N316R</sub>. Although we have not examined the DNA-binding properties of the N316 variants and thus could not attribute the altered in vivo regulatory modes only to their anomalous oligomerization properties, it is established that the N316 residue is crucial for the protein to fine-tune its oligomeric state in response to the ligands. It should be noted that the amino acid residues at this position vary among the other NtrC family members (A in <em>E. coli</em> NtrC, Q in <em>Salmoneilla enterica</em> serovar Typhimurium ZraR and <em>Klebsiella pneumoniae</em> NifA, and E in <em>Sinorhizobium meliloti</em> DetD, <em>E. coli</em> PsfE, and <em>Aquifex aeolicus</em> NtrC1) and that the corresponding residue of <em>A. aeolicus</em> NtrC1 (E256) forms a hydrogen bond with the sensor II residue from a neighbor subunit (PDB code 1NY6) (13). The sensor II residue is thought to be critical for discrimination of ATP/ADP-bound forms. Considering the oligomer formation of TyrR<sub>N316D</sub> in the presence of ADP and Phe or Tyr, it is interesting to speculate that the role of N316 is as a sensor II modulator, although the interaction of N316 and the sensor II residue (R417) of <em>E. coli</em> TyrR is not established. Further in vitro studies are required to elucidate the role of the residue in the oligomer formation process of TyrR.

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