The Central Domain of *Escherichia coli* TyrR is Responsible for Hexamerization Associated with Tyrosine-Mediated Repression of Gene Expression*

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*This work was supported by the Australian Research Council Large Grant A09930002 (to B.E.D.)

§ In memory of the late Professor Barrie Davidson.

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Running title: Hexamerization of the Central Domain of *E. coli* TyrR
SUMMARY

TyrR from *Escherichia coli* regulates the expression of genes for aromatic amino acid uptake and biosynthesis. Its central ATP-hydrolysing domain is similar to conserved domains of bacterial regulatory proteins that interact with RNA polymerase holoenzyme associated with the alternative sigma factor, $\sigma^{54}$. It is also related to the common module of the AAA+ superfamily of proteins that is involved in a wide range of cellular activities. We expressed and purified two TyrR central domain polypeptides. The fragment comprising residues 188 to 467, called TyrR(188-467), was soluble and stable, in contrast to that corresponding to the conserved core from residues 193 to 433. TyrR(188-467) bound ATP and rhodamine-ATP with association constants 2 to 5-fold lower than TyrR and hydrolyzed ATP at five times the rate of TyrR. In contrast to TyrR, which is predominantly dimeric at protein concentrations less than 10 $\mu$M in the absence of ligands, or in the presence of ATP or tyrosine alone, TyrR(188-467) is a monomer, even at high protein concentrations. Tyrosine in the presence of ATP or ATP$\gamma$S promotes the oligomerization of TyrR(188-467) to a hexamer. Tyrosine-dependent repression of gene transcription by TyrR therefore depends on ligand binding and hexamerization determinants located in the central domain polypeptide TyrR(188-467).
INTRODUCTION

TyrR plays a central role in the regulation of aromatic amino acid biosynthesis and transport (for reviews, see (1,2)). In *E. coli*, it represses or activates the expression of at least eight unlinked operons in response to aromatic amino acid levels. TyrR binds to sites called TyrR boxes with the palindromic consensus sequence \(N_2\)TGTAAN\(_6\)TTTACAN\(_2\) in the promoters of these operons, of which there are two classes. Strong boxes are very similar to the consensus sequence, and bind TyrR in vitro in the absence of aromatic amino acids. Weak boxes have lower similarity to the consensus sequence, and require an adjacent strong box, ATP, and tyrosine to bind TyrR. TyrR binding to weak boxes represses transcription.

TyrR is a protein of 513 amino acids that is homodimeric at low protein concentrations in the absence of ligands. Amino acid sequence comparisons and limited proteolysis indicate that TyrR consists of three domains (3,4). The N-terminal domain is similar to only one known protein, PhhR, which regulates phenylalanine hydroxylase and the phenol degradation pathway in *Pseudomonas Sp.* (5,6). The N-terminal domain contains an ATP-independent aromatic amino acid binding site. The interaction of this domain with aromatic amino acids and RNA polymerase is thought to mediate transcriptional activation (7,8). The C-terminal domain contains a helix-turn-helix DNA-binding motif (1,9). When cloned and expressed independently the N and C-terminal domains were found to be dimeric (10). The central domain is similar to the 230 amino acid conserved ATP-binding domains of the sigma 54-interaction family, which includes NtrC and NifA that are involved in nitrogen and nitrogenase regulation respectively (Fig. 1). Members of this protein family regulate genes transcribed by RNA polymerase holoenzyme associated with the alternative sigma factor, \(\sigma^{54}\) (1,3). However, operons regulated by TyrR have promoters recognized by RNA polymerase holoenzyme associated with the major sigma factor, \(\sigma^{70}\). The central domain is also related to the large superfamily of AAA+ proteins that have wide-ranging functions based on a common ATPase structural module (11,12).
ATP enhances the affinity of the TyrR dimer for DNA (13). It is also required for aromatic amino acids to bind to a second site within TyrR (7). TyrR undergoes self-association from a dimer to a hexamer when tyrosine, the major effector of repression, binds to this ATP-dependent site (7,14). This ligand-induced oligomerization explains the co-operative binding of TyrR to the strong and weak boxes in operons that are repressed in response to tyrosine (14). ATP binding, aromatic amino acid binding, and self-association are therefore associated with tyrosine-mediated repression. In this study we isolated a soluble polypeptide encompassing the central domain of TyrR, and investigated its ability to bind ATP, ATP analogues, aromatic amino acids, and to self-associate. We show that the central domain of TyrR uses functions common to AAA+ proteins to effect repression of gene transcription.
EXPERIMENTAL PROCEDURES

Plasmids Encoding TyrR and Central Domain Fragments--- Plasmid pMU1082 contains the E. coli tyrR gene cloned into pET-3a (15). Plasmid pMU1090 contains the E. coli tyrR gene excised from pMU1082 and inserted into the XbaI and BamHI sites of pET-11a (Novagen). Plasmid pMU5612, the plasmid used to express amino acids 188 to 467 of TyrR with a C-terminal affinity tag, was constructed using standard methods by inserting the appropriate DNA sequence amplified by the PCR from plasmid pMU1090 into the NdeI and XhoI sites of the vector pET-22b (Novagen). Plasmids pMU5610 and pMU5611, which express TyrR fragments from amino acids 193 to 432 with N and C-terminal His	extsubscript{6} tags respectively, were constructed by inserting DNA sequences PCR-amplified from pMU1090 into the NdeI and BamHI sites of the vectors pET-15b (Novagen) and pET-22b respectively.

Protein Expression and Purification--- E. coli BL21(DE3) carrying the plasmids pMU5612, pMU5610, or pMU5611 were grown in 4 x 500 ml 2 x YT medium containing 200 µg/ml ampicillin at 37 °C in 2-litre flasks with rotary shaking. Protein expression was induced by addition of 1 mM isopropyl-1-thio-D-galactopyranoside when the absorbance of the culture at 600 nm reached 0.6. Cells were harvested by centrifugation 2 h after induction. They were resuspended in 10 mM Tris-HCl, pH 8.0, 100 mM KCl, 2 mM EDTA, 10 % glycerol, 5 mM imidazole at 4 °C, and lysed by sonication. The lysed cells were centrifuged at 20,000 × g for 40 min at 4 °C. The cell-free extract, supplemented with 10 mM MgCl	extsubscript{2}, was applied to a 5 ml column of TALON metal affinity resin (Clontech) equilibrated with lysis buffer. Unbound proteins were removed with several volumes of the lysis buffer, and the His	extsubscript{6}-tagged proteins eluted in the same buffer containing 80 mM imidazole. The proteins were concentrated using 10 kDa molecular weight cut-off centrifugal concentrators and further purified by gel filtration at 4 °C using a Pharmacia HR 16/50 Superose 12 FPLC column run in 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM DTT, 1 mM EDTA. Thrombin cleavage of the N-terminal His	extsubscript{6} tag of TyrR(193-432) was carried out at 20 °C with 1 unit thrombin/mg protein in 20 mM
Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM CaCl₂. The tagged central domain fragments encompassing amino acids 193-432 and 188-467 are referred to as TyrR(193-432) and TyrR(188-467) respectively.

TyrR was purified as described previously except that it was expressed from pMU1090 in *E. coli* BL21(DE3) instead of from pMU1082 in *E. coli* HMS174 (15,16).

**Protein Analysis**--- Proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels stained with Coomassie Blue R250. Native protein concentrations were determined by absorbance. The previously determined extinction coefficient at 280 nm of 34,470 × M⁻¹ cm⁻¹ (monomer) was used for TyrR (17). Extinction coefficients of 14,440 × M⁻¹ cm⁻¹ at 280 nm for both N and C-terminal His₆- tagged TyrR(193-432) were calculated from the amino acid compositions using the program SEDNTERP (18). The extinction coefficients for TyrR(188-467) were experimentally determined as 17,420 × M⁻¹ cm⁻¹ at 280 nm, and as 6.12 × 10³ M⁻¹ cm⁻¹ at 290 nm using the method described previously (17).

**Gel Filtration**--- The effect of tryptophan on the apparent molecular weight of TyrR(188-467) was determined by gel filtration chromatography on Superose 12 in 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM DTT, with or without 25 mM tryptophan and 200 µM ATP, by reference to a calibration curve obtained with proteins of known molecular weight. The elution volume of TyrR(188-467) was determined by measuring the absorbance at 595 nM of 100 µl aliquots of 1 ml fractions with the protein assay (BioRad) based on the method of Bradford (19).

**Mass Spectrometry**--- Mass analysis was carried out using a Bruker Esquire 3000+ ion-trap mass spectrometer (Bruker Daltonics) equipped with an electrospray ionization source. Samples were prepared with 1 volume of 50 % methanol/0.1 % formic acid. Samples were introduced at 3 µl/min. with a spray needle voltage of 4200 volts, a capillary temperature of 350 °C, and nitrogen drying gas flow at 7 l min⁻¹.

**Circular Dichroism (CD) Spectropolarimetry**--- CD spectra were obtained with an AVIV 62DS CD spectrophotometer with a 400 µl 2 mm path-length quartz cell. The spectrum was measured from
190 nm to 250 nm in 0.5 nm intervals at 20 °C. Data was smoothed with a 3-point moving window. The secondary structure of TyrR(188-467) was estimated using the CDSSTR program (20). Secondary structure was calculated by comparison with a reference set of 29 proteins.

**ATPase Assay**---The ATPase activity was determined in 100 µl reactions containing 200 µM ATP, 2 nM [γ\(^{32}\)P]ATP (4000 Ci mmol\(^{-1}\)), 10 mM Tris-HCl, pH 7.0, 10 mM MgCl\(_2\), 100 mM KCl. Reactions were initiated by the addition of 9 µM TyrR or TyrR central domain polypeptides. Reaction mixtures were incubated at 37 °C. Samples of 1 µl were taken at 15 min intervals up to 1.8 h and applied to polyethyleneimine thin-layer chromatography plates (Merck). The TLC plates were dried before development in 0.5 M LiCl\(_2\), 1 M CH\(_3\)COOH. The relative quantities of resolved inorganic phosphate and ATP were determined using a phosphorimager (Molecular Dynamics).

**ATP Binding Assay**---ATP binding was determined by flow dialysis (21). Experiments were carried out in 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM DTT, 5 mM MgCl\(_2\) at 20 °C using a flow dialysis apparatus with 800 µl upper and lower chambers and a 2 cm\(^2\) diffusion area. 5 ml fractions were collected at a flow rate of 8 ml min\(^{-1}\). 2 nM [γ\(^{33}\)P]ATP (4000 Ci mmol\(^{-1}\)) was added to the chamber containing 40 µl TyrR or TyrR(188-467) prior to titration by addition of successive 5–10 µl aliquots of unlabelled ATP (typically 1–10 mM). Transfer of ATP across the membrane was determined by scintillation counting.

**Fluorescence Spectroscopy**---Fluorescence anisotropy measurements were carried out using a SPEX Fluorolog Tau-2 instrument as described previously (22). Fluorescence anisotropy measurements were made in the L-format with excitation and emission wavelengths of 560 and 600 nm respectively. All experiments were carried out at 20 °C in 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM DTT, 5 mM MgCl\(_2\), with 20 nM rhodamine-ATP.

**Sedimentation Equilibrium Analysis**---Sedimentation equilibrium experiments were performed in an Optima XL-A analytical ultracentrifuge (Beckman) using absorbance optics at 290 nm. Centrifuge cells in a Ti60 rotor were fitted with two channel or six channel 12 mm pathlength center-pieces.
ATPγS was used in place of ATP as described previously (14). Centrifugation was carried out at 20 °C. Radial scans were performed at 1 h intervals. Equilibrium was normally attained after 14 h when successive scans were superimposable. A high-speed experiment (40,000 rpm) was then performed to deplete protein to determine the baseline absorbance in each cell. Samples were prepared in 20 mM Tris-HCl, 100 mM KCl, 1 mM DTT, 1 mM EDTA, and ligands added as described below. Data analysis according to self-association models used the following general equation:

\[
B = c_{l,r} = c_{P,r_0}e^{\omega^2 2RT \left(1 - \frac{1}{n} \right)}(r^2 - r_0^2) + K_{P-N} (c_{P,r_0})^n e^{\omega^2 2RT nM \left(1 - \frac{1}{n} \right)}(r^2 - r_0^2) + B
\]

where \(c_{l,r}\) is the total protein concentration at radius \(r\) in terms of extinction coefficient, \(c_{P,r_0}\) is the concentration of protomer at the reference radius \(r_0\), \(\omega\) is the angular velocity in radians/sec., \(\rho\) is the density of the solution in g/ml (\(\rho = 1.007\) (14)), \(v\) is the partial specific volume of the protein, \(R\) is the gas constant, \(T\) is the absolute temperature, \(M\) is the molecular weight of the protomer, \(B\) is a baseline correction for absorbance due to the sample buffer, \(n\) is the stoichiometry of the oligomer (23). \(K_{P-N}\) is the association constant describing the protomer-oligomer equilibrium in terms of all the species and is defined as:

\[
K_{P-N} = \frac{c_N}{(c_p)^n}
\]

where \(c_N\) is the concentration of the oligomer and \(c_p\) is the concentration of the protomer. The sedimentation equilibrium data were collected as absorbance data and the resulting association constants were therefore in absorbance units. The molar association constants, \(K_{P-N(molar)}\) given in the text were calculated from association constants in the absorbance form, \(K_{P-N(\text{absorbance})}\), according to equation:

\[
\text{(equation)}
\]
\[ K_{P-N (molar)} = K_{P-N (absorbance)} \frac{(\varepsilon \times l)^{n-1}}{n} \]

where \( \varepsilon \) is the extinction coefficient at the wavelength of the experiment and \( l \) is the path-length of the centrifuge cell (24). Sedimentation data was analyzed using the program XLAEQ (Beckman) and non-linear least-squares curve fitting performed with the program SigmaPlot (Jandel Scientific).

*Dynamic Light Scattering*--- Dynamic light scattering measurements were performed using an Autosizer 4700 light scattering spectrometer (Malvern Instruments) equipped with a 10 mW Ar\(^{+}\) ion laser (488 nm). Measurement was performed at an angle of 90\(^\circ\) and a temperature of 25 \(^\circ\)C. 500 \( \mu \)l protein samples were prepared in 25 mM KH\(_2\)PO\(_4\)-K\(_2\)HPO\(_4\), pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT, and ligands as described in the text. TyrR(188-467) concentrations were 40, 76, 255, and 715 \( \mu \)M in the absence of ligands. Data analysis was carried out as described previously (25).
RESULTS

Central Domain Polypeptides--- We purified two polypeptides containing the central domain as defined by amino acid sequence analysis and proteolysis. The polypeptide spanning amino acids 193 to 433 was purified with both N and C-terminal His$_6$ tags. Both polypeptides were less than 50 % soluble. Gel filtration of the soluble protein revealed several species with molecular weights corresponding to monomer, dimer, trimer, and higher molecular weight oligomers (data not shown). The proportion of high molecular weight species increased during storage at 4 °C. None of the lower molecular weight species were observed when the higher molecular weight species were subject to gel filtration for a second time. This suggests that a soluble monomeric polypeptide was undergoing non-reversible aggregation rather than an equilibrium distribution between different oligomeric states. Removal of the N-terminal tag by thrombin cleavage increased precipitation. The purified polypeptides were also sensitive to proteolytic cleavage during purification. A polypeptide comprising amino acids 188 to 467 with a C-terminal tag was then produced. This was expressed with a yield of approximately 60 mg protein per liter of culture and did not undergo proteolytic degradation during purification. TyrR(188-467) in 25 mM KH$_2$PO$_4$, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM DTT remained soluble for at least 2 weeks at concentrations greater than 0.5 mM (16 mg/ml). Gel filtration revealed a single species. The identity of TyrR(188-467) was confirmed by ion-trap mass spectrometry. The observed peak of 31,876 is identical to the mass of TyrR(188-467) with the N-terminal methionine cleaved. Circular dichroism spectroscopy estimated the secondary structure to be 22 % α–helix, 28 % β–sheet, 50 % remainder (data not shown). This is compared with 46 % α–helix, 11 % β–sheet, and 43 % remainder predicted using the PHD method of secondary structure prediction (26). The estimate of secondary structure from the CD data is limited by the lack of short wavelength data.

ATPase Activity, ATP and Rhodamine-ATP Binding---TyrR possesses weak ATPase activity that is variously reported as being between 12 and 400 mmol of ATP mol$^{-1}$ monomer min$^{-1}$ (16,27).
TyrR(188-467) had a specific ATPase activity of 105 mmol ATP mol\(^{-1}\) monomer min\(^{-1}\), approximately 5-fold the value for TyrR which was found in this study to be 20 mmol ATP mol\(^{-1}\) monomer min\(^{-1}\). The TyrR polypeptides from amino acids 190 to 433 with both N and C-terminal His\(_6\) tags were also assayed for ATPase activity. They both hydrolyze ATP at a similar rate to TyrR(188-467), though this may not be comparable as the proteins have multiple aggregating species. Flow dialysis showed that TyrR bound ATP with a half saturation value 3.1 µM (Fig. 2). TyrR(188-467) bound ATP with a half saturation value of 7.6 µM. The binding of the ATP analogue rhodamine-ATP was determined by measuring the increase of its fluorescence anisotropy on binding TyrR and TyrR(188-467) (Fig. 3). The dissociation constant of 0.28 µM determined for TyrR agrees with the value of 0.3 µM previously obtained using this technique (22). TyrR(188-467) bound to Rhodamine-ATP with a dissociation constant of 1.4 µM. These analyses show that TyrR(188-467) bound both ATP and rhodamine-ATP with 2 to 5-fold lower affinity than TyrR. These analyses also confirm that the concentration of ATP used in the ATP hydrolysis assay is likely to approach saturating and the specific ATPase activity therefore represents maximal ATPase velocity. A previous study showed that the dissociation constant for ATP binding to TyrR was approximately the same as Km (28). TyrR(188-467) contains a single tryptophan at amino acid 412. The fluorescence emission intensity of TyrR(188-467) at 350 nm was unchanged in the presence of either 200 µM ATP, or 200 µM ATP and 1 mM tyrosine.

*TyrR(188-467) is a Monomer---* TyrR exists in a dimer–hexamer equilibrium, with hexamer formation favored in the presence of a combination of ATP and an aromatic amino acid. To determine what role the central domain plays in the association states of TyrR, the self-association of TyrR(188-467) was investigated by sedimentation equilibrium analysis. Sedimentation equilibrium data obtained with 18 µM TyrR(188-467) were well described by a model for a single homogeneous species with a molecular mass of 31-kDa (Fig. 4). Data obtained in the presence of any one of 200 µM ATP\(\gamma\)S, 25 mM phenylalanine, or 1 mM tyrosine were also well described by a model for a single homogeneous species, with molecular masses of 31, 32, and 33-kDa respectively (Fig. 4). Attempts to
fit these data to equilibrium models involving monomer and higher molecular weight species predicted extremely small proportions of higher molecular weight species. This analysis shows that the central domain is a monomer, in contrast to TyrR that is a dimer in a concentration-dependent equilibrium with a hexamer. Dynamic light scattering was employed to investigate the association states of TyrR(188-467) at protein concentrations higher than can be used in ultracentrifugation (Fig. 5). TyrR(188-467) at protein concentrations of 40 to 715 µM was a single species with a hydrodynamic diameter of 5.3 ± 0.7 nm. A 475 µM sample of TyrR(188-467) containing 1 mM ATPγS, 5 mM MgCl2 had a mean diameter of 5.2 nm.

TyrR(188-467) Self-associates to a Hexamer in Response to ATP-dependent Aromatic Amino Acid Binding--- In the presence of 1 mM tyrosine and 200 µM ATPγS, the sedimentation profile of TyrR(188-467) at concentrations of 8 µM, 18 µM, and 54 µM was consistent with a self-associating system (Fig. 6). Monomer-hexamer or monomer-dimer-hexamer models both fitted the data well, however, it was not possible to differentiate between these two models. The association constant was $3.5 \times 10^{23} \text{M}^{-5}$ for a monomer-hexamer equilibrium. This association constant reflects TyrR(188-467) being approximately 50 % hexameric at 50 µM. The association constants for the three-step model predicted very small amounts of dimeric intermediate. The association constants were: $K_{M-D} = 9.1 \times 10^2 \text{M}^{-1}$ and $K_{D-H} = 2.5 \times 10^9 \text{M}^{-2}$. Monomer-dimer, -trimer, -octomer, and dimer-tetramer, -hexamer models fitted poorly. Sedimentation equilibrium experiments carried out in the presence of 25 mM phenylalanine and 200 µM ATPγS also indicated a monomer-hexamer equilibrium (Fig. 6).

Dynamic light scattering was also used to investigate the molecular size of TyrR(188-467) at a high protein concentration (357 µM) in the presence of 1 mM tyrosine and 200 µM ATPγS (Fig. 5). Under these conditions TyrR(188-467) was a single species with a hydrodynamic diameter of 9.6 nm compared with 5.3 nm in the absence of tyrosine. The near doubling of the monomer diameter is consistent with the formation of a species with a six to seven-fold increase in volume.
Competition of tryptophan for tyrosine bound to the ATP-dependent aromatic amino acid binding site in TyrR suggested that tryptophan induced self-association (7). We used gel filtration chromatography to determine the effect of tryptophan on the association state of TyrR(188-467) as tryptophan absorbance interferes with the detection of protein by UV absorbance in the ultracentrifuge. The presence of 25 mM tryptophan and 200 µM ATP changes the elution volume of TyrR(188-467) from a value consistent with a monomer to one similar to that observed with 1 mM tyrosine and 200 µM ATP (data for tyrosine not shown) (Fig. 7). The concentration of tryptophan used was assumed to be saturating since the dissociation constant for tryptophan to the ATP-dependent binding site in TyrR is 10 mM (7).

Self-association of TyrR(188-467) Depends on Tyrosine Concentration --- To determine the affinity of TyrR(188-467) for tyrosine, we investigated the effect of tyrosine concentration on self-association by equilibrium analytical ultracentrifugation. At each tyrosine concentration the data was fitted to an equation describing a monomer-hexamer model as described above (Fig. 8). The half-maximal saturation value was determined to be 300 µM for TyrR(188-467) compared with 250 µM for TyrR (14).
DISCUSSION

The central domain of TyrR links it both structurally and functionally with the large family of bacterial transcriptional regulators typified by NtrC and NifA (1,3). Current methods for recognizing protein sequence homologues using profile analysis show that it is also related to the AAA+ superfamily, that includes the established AAA family of proteins (11,29). Both amino acid sequence alignments and proteolysis place the N-terminal boundary of the central domain at about amino acid 207. A poorly conserved glutamine-rich region called the Q-linker, from amino acids 190 to 206, separates the central domain from the N-terminal domain (Fig. 1) (30). Limited tryptic proteolysis of E. coli TyrR cleaves between Arg190 and Glu191 near the beginning of the Q-linker (4). The C-terminal boundary is less clearly defined. Sequence alignment places it at approximately amino acid 432 giving a 27-kDa fragment that corresponds to a core domain suggested by sequence analysis using folding algorithms (31). A similar core fragment was identified in E. coli NtrC by tryptic proteolysis (32). On the other hand, limited tryptic proteolysis of E. coli TyrR gives a 31-kDa central domain polypeptide with the C-terminus at Arg467. Recently, limited proteolysis combined with mass spectrometry identified a linker called the PASE-linker located beyond the highly conserved core region of NtrC and immediately before the region that forms the four-helix bundle of its C-terminal domain (32,33). Sequence alignment shows that TyrR has a region that corresponds to the PASE-linker. It is poorly conserved as expected of linkers, and rich in alanine and acidic residues as in the PASE linker, though aspartic acid residues predominate instead of glutamic acid (Fig. 1). Limited tryptic proteolysis cleaves at the first possible site C-terminal to this region, and not at possible sites that immediately precede it. The solution structure of the C-terminal domain of H. influenzae has been determined, and there is 48 % sequence identity between it and the corresponding fragment of E. coli TyrR (34). A homology model (not shown) based on the H. influenzae solution structure predicts that C-terminal tryptic cleavage site in E. coli TyrR is near the start of the first of the three well defined helices in the C-terminal domain (34).
We studied the two distinct fragments defined by amino acid alignment and proteolysis. The 27-kDa polypeptide TyrR(193-433), corresponding to the conserved core, aggregates irreversibly. By contrast, the 32-kDa fragment, TyrR(188-467) that extends from the Q-linker to beyond the PASE-linker region is mono-disperse and soluble. The amino acids beyond the highly conserved region clearly stabilize it. TyrR is a weak ATPase and both TyrR central domain fragments retain this function. The affinities of TyrR and the isolated central domain for tyrosine are comparable. Removal of the N and C-terminal domains however, results in a significant increase in ATPase activity accompanied by a 2-3 fold decrease in binding affinity. How the removal of the N and C-terminal domains changes the kinetics of ATP binding and hydrolysis remains unexplained. It is however likely to must reflect interactions between the terminal domains and the central domain or alternatively a pair of central domains within the TyrR dimer. Cooperativity of ATP binding has been reported for TyrR, providing further evidence for communication between the central domains of TyrR dimers (15).

A number of observations indicate that ATP binding produces important conformational changes. ATP protects a central domain fragment comprising amino acids 191 to 467 from cleavage at Arg398 by high concentrations of trypsin (4). ATP has a major effect on the unfolding of the central domain of *H. influenzae* TyrR (35). Mutations in the ATP-binding motif of *E. coli* TyrR produce a protein that is unable to repress tyrosine-repressible genes and is defective in tyrosine-induced hexamerization (36). The conformational change produced by ATP presumably increases the affinity of the aromatic amino acid binding site in the central domain.

TyrR is a dimer in a concentration-dependent equilibrium with a hexamer in the absence of ligands or in the presence of a single ligand. Consequently 1 µM TyrR is predominantly a dimer and 700 µM TyrR is almost 100 % hexameric in the presence of ATPγS alone (37). It was therefore surprising to find by light scattering that in the absence of ligands TyrR(188-467) remains monomeric at concentrations up to 715 µM. Fluorescence anisotropy shows that the homologous domain of *H. influenzae* TyrR is similarly monomeric at low protein concentration, but in contrast to *E. coli* TyrR it associates to a dimer in the presence of ATP (38). The effect of tyrosine was not reported.
Aromatic amino acids in the presence of ATP shift the equilibrium of TyrR from a predominantly dimeric to hexameric form (14). TyrR is 50% hexameric at 110 \( \mu M \) in the presence of ATP\( _\gamma \)S alone whereas it is 50% hexameric at 220 nM in the presence of ATP\( _\gamma \)S and tyrosine. We provide direct evidence that the hexamerization of TyrR depends on determinants in the central domain polypeptide TyrR(188-467). Furthermore hexamerisation of the isolated central domain does not appear to involve a significant dimeric intermediate. Since earlier studies in our laboratory showed that the isolated N and C-terminal domains are dimeric, we conclude that the terminal domains effect dimerization of TyrR (10). However one or both of these domains also influences the hexamerization brought about by the central domain, as hexamers are more readily formed from TyrR than the isolated central domain. TyrR(188-467) is 50% hexameric at 50 \( \mu M \) in the presence of ATP\( _\gamma \)S and tyrosine compared with TyrR which is 50% hexameric at only 500 nM in the presence of the same ligands. As in many AAA proteins, hexameric TyrR would be assembled by interaction of interfaces of the ATPase domain. Hexamers assemble by interaction of interfaces of the ATPase domain, whether by the hexamerization of isolated central domain monomers or by trimerisation of TyrR dimers. AAA proteins typically form hexameric rings by head-to-tail packing of their triangular shaped \( \alpha/\beta \) domains in response to ATP and ligands (12,39). Although ATP alone does not effect oligomerization it does induce a conformational change which facilitates tyrosine binding, which in turn results in a further change favoring the formation of hexamers. ATP appears to stabilize the hexameric interactions of the AAA protein N-ethylmaleimide-sensitive fusion protein (NSF) (40).

High concentrations of phenylalanine and tryptophan are required for hexamerization in vitro. These amino acids would therefore not be expected to bring about significant hexamerization at physiological concentrations. By contrast, hexamerization is promoted by micromolar concentrations of tyrosine, the major co-repressor of the tyrR regulon. The detailed mechanism of repression by TyrR varies in different operons. Almost all cases of tyrosine-mediated repression involve TyrR binding to a strong and adjacent weak box, with consequent hindrance of one of the steps of transcription initiation.
(2). In the absence of tyrosine, a TyrR dimer with bound ATP would occupy a strong box. Increasing tyrosine concentration would promote hexamerization, and binding to the adjacent weak box.

The wider interest in studying the isolated ATPase domain is to identify how features common to the widespread AAA+ superfamily of proteins are used in the important class of bacterial transcriptional regulators to which TyrR belongs. Oligomerization plays an important role in regulation by many proteins containing the sigma 54 interaction domain (41-45). In NtrC it is induced by phosphorylation of the N-terminal domain and required for ATPase activity that is coupled to transcriptional activation (46). NtrC is believed to be in a dimer-octamer or -hexamer equilibrium in the presence of DNA (42,47). ATPγS causes a mutant NtrC that activates transcription in the absence of phosphorylation to oligomerize to a hexamer or octamer in the absence of DNA (48). The major dimerisation determinants of NtrC are located in its carboxy-terminal domain, and indirect evidence points to oligomerization determinants being located in the central domain (46,48). In contrast to NtrC, the ligand binding sites that promote the higher order oligomerization of TyrR are located solely within the central domain. In both NtrC and TyrR, oligomerization provides a means of recruiting multiple DNA binding sites to form a stable protein-DNA complex with possibly altered DNA structure. Despite the striking differences in the functioning of NtrC and TyrR, both have harnessed oligomerization, ATP binding, and ligand binding to regulate transcription in different ways. The precise signal-induced oligomerization of its AAA-like module switches TyrR from a transcriptional activator to repressor.

Acknowledgements---We are grateful to Paul Gooley, James Swarbrick, Isobel Lawrenson, and Jim Pittard for helpful discussions. We are also grateful to John Eccleston for generously providing the Rhodamine-ATP, to Paul O’Donnell for assistance with mass spectrometry, and Robert Chan for assistance with circular dichroism spectroscopy. M.P.D. was supported by a Melbourne Research Scholarship.
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Abbreviations

The abbreviations used are: ATPγS, adenosine 5'-O-(3-thiotriphosphate); DTT, dithioerythritol; $K_{M-D}$, $K_{M-H}$, $K_{D-H}$, association constants for monomer-dimer, monomer-hexamer, and dimer-hexamer equilibrium respectively.
Fig. 1. Amino acid sequence alignments between E. coli TyrR and related proteins. The sequence containing the central and C-terminal domains of E. coli TyrR is aligned with the sequences of 6 members of the sigma 54 interaction family and the AAA protein NSF (N-ethmaleimide-sensitive fusion protein). The alignment was performed with the program MULTALIGN (49). Amino acids that are identical and similar to those in E. coli TyrR are shown on black and grey backgrounds respectively. The dots indicate 10 amino acid intervals in TyrR. The sigma 54 interaction domain in TyrR extends from amino acids 206-428. The central domain defined by proteolytic cleavage extends between the tryptic cleavage sites shown by arrows 1 and 2 (4). Arrow 3 indicates the trypsin cleavage site protected by ATP. The boxes designate the Q-linker of TyrR, the Walker A and B ATP-binding motifs, the PASE-linker of NtrC (32), and the helix-turn-helix motif based on the structure of the C-terminus of H. influenzae TyrR (34). The names used follow the SWISS-PROT naming convention. TYRR_ECOLI is TyrR E. coli (P07604), TYRR_HAEIN is TyrR Haemophilus influenzae (P44694), PHHR_PSEPU is PhhR Psedomonas putida (Q52177), NIFA_AZOB is NifA Azotobacter Vinelandii (P09570), XYLR_PSEPU is XylR Psedomonas putida (P21940), PILR_PSEAE is PilR Psedomonas aeruginosa (Q00934), NTRC_ECOLI is NtrC E. coli (P06713). Swiss-Prot or TrEMBL database accession numbers are given in brackets.

Fig. 2. Flow dialysis analysis of ATP binding to TyrR and TyrR(188-467). The fractional saturation with ATP of 40 µM TyrR (closed circles) and TyrR(188-467) (open circles) at various concentrations of ATP. The solid lines represent the least squares best fits with resulting dissociation constants of 3.1 µM for TyrR and 7.6 µM for TyrR(188-467).

Fig. 3. Fluorescence anisotropy analysis of nucleotide binding to TyrR and TyrR(188-467). The fluorescence anisotropy of 20 nM rhodamine-ATP at various concentrations of TyrR (closed circles) and TyrR(188-467) (open circles). The solid lines represents the least squares best fits with resulting dissociation constants of 280 nM for TyrR and 1.4 µM for TyrR(188-467).
**Fig. 4.** Sedimentation equilibrium analysis of TyrR(188-467). The absorbance of TyrR(188-467) at 290 nm at sedimentation equilibrium plotted versus the distance from the axis of rotation. For clarity, only every third data point is shown. The solid lines represent the non-linear least squares best fits to a single species model. The upper panels show the corresponding residuals. Samples with an initial protein concentration of 18 μM were sedimented at 15,000 rpm (A, B, and C) and 12,000 rpm (D) for 20 h. TyrR(188-467) in the absence of ligands (A), in the presence of 200 μM ATPγS, 5 mM MgCl₂ (B), 1 mM tyrosine (C), and 25 mM phenylalanine (D).

**Fig. 5.** Size distribution of TyrR(188-467) determined by dynamic light scattering. 500 μM TyrR(188-467) in the absence of ligands (solid line), 475 μM TyrR(188-467) in the presence of 200 μM ATPγS (dotted line), and 357 μM TyrR(188-467) in the presence of 200 μM ATPγS and 1 mM tyrosine (broken line).

**Fig. 6.** Sedimentation equilibrium analysis of TyrR(188-467) in the presence of ATPγS and either tyrosine or phenylalanine. The absorbance at 290 nm at sedimentation equilibrium plotted versus the distance from the axis of rotation. Only every third data point is shown. The solid lines represent the global non-linear least squares best fits to a monomer-hexamer model. The upper panels show the corresponding residuals. (A) TyrR(188-467) with initial protein concentrations of 8 μM (squares), 18 μM (open circles), and 54 μM (closed circles) in the presence of 1 mM tyrosine, 200 μM ATPγS, 5 mM MgCl₂, sedimented at 15,000 rpm for 20 h. (B) TyrR(188-467) with an initial concentration of 18 μM in the presence of 25 mM phenylalanine, 200 μM ATPγS, 5 mM MgCl₂, sedimentated at 12,000 rpm for 20 h (open circles), and then at 18,000 rpm for a further 20 hours (closed circles).
Fig. 7. The effect of tryptophan and ATP on the molecular weight of TyrR(188-467) analysed by gel filtration chromatography. TyrR(188-467) was chromatographed in 25 mM potassium phosphate, pH 7.5, 100 mM KCl, 1 mM DTT (closed circles), and the same buffer containing 25 mM tryptophan, 200 µM ATP, 5 mM MgCl₂ (open circles). The absorbance of aliquots of fractions assayed for protein content is plotted versus the elution volume.

Fig. 8. The effect of tyrosine on the self-association of TyrR(188-467). The association constants for a model describing a monomer-hexamer equilibrium were determined by equilibrium analytical ultracentrifugation at 20,000 rpm for 20 h with 16 µM TyrR(188-467) in the presence of 200 µM ATPγS, 5 mM MgCl₂, and tyrosine at concentrations up to 1 mM. The best fits yielded association constants $K_{M-H}$ which are plotted as Log₁₀ values against tyrosine concentration. The half-maximal saturation of TyrR(188-467) is approximately 300 µM tyrosine.
Figure 1.

Walker A

Walker B

PASE-Linker

Helix-turn-Helix
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.

[Graph showing absorbance at 595 nm against elution volume in ml.]
Figure 8.
Equations

\[
c_{t,r} = c_{P,r_0} e^{\left[ \frac{\omega^2}{2RT} M (1 - \nu \rho) (r^2 - r_0^2) \right]}
+ K_{p-N} (c_{P,r_0})^n e^{\left[ \frac{\omega^2}{2RT} nM (1 - \nu \rho) (r^2 - r_0^2) \right]} + B
\]

\[
K_{p-N} = \frac{c_N}{(c_P)^n}
\]

\[
K_{p-N\text{(molar)}} = K_{p-N\text{(absorbance)}} \frac{(\varepsilon \times l)^{n-1}}{n}
\]
The central domain of Escherichia coli TyrR is responsible for hexamerization associated with tyrosine-mediated repression of gene expression
M. P. Dixon, R. N. Pau, G. J. Howlett, D. E. Dunstan, W. H. Sawyer and B. E. Davidson
J. Biol. Chem. published online March 28, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M112184200

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