The influence of ATP on the binding of aromatic amino acids to the ligand response domain of the tyrosine repressor of *Haemophilus influenzae*

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Abstract The binding of aromatic amino acids to the ligand response domain of the tyrosine repressor (TyrR) protein ($TyrR_{lrd}$) of *Haemophilus influenzae* was investigated using circular dichroism and fluorescence spectroscopy. The induced secondary structural changes were unique for each aromatic amino acid and were further influenced by the presence or absence of ATP. Tyrosine was found to have the highest affinity for $TyrR_{lrd}$ in the absence of ATP, whereas the affinity for ATP itself increased in the presence of tyrosine. Binding of tyrosine is therefore the conformational trigger for the activation of TyrR whereas ATP is regarded as a conformational co-activator.

Key words: Transcriptional regulation; Ligand binding; Tyrosine repressor; Fluorescence spectroscopy; Circular dichroism

1. Introduction

The tyrosine repressor (TyrR) proteins belong to the NtrC superfamily of prokaryotic transcription factors. They control the expression of several genes involved in the biosynthesis and transport of aromatic amino acids [1,2]. Their exact mode of action remains unclear because of the ability of these proteins to up- or down-regulate the transcription of their target genes. TyrR of *Escherichia coli* (513 amino acid residues) consists of three domains, the N-terminal, the central and the DNA binding domain, and acts on its operator sequences as a homo-dimer [3,4]. The central domain (amino acids 191–467) contains the tyrosine binding site as well as at least one binding site for ATP. Although TyrR was reported to show a weak ATPase activity [5], the exact role of ATP has not yet been clarified.

Based upon limited proteolytic cleavage, the TyrR protein of *Haemophilus influenzae* was found to consist of only two domains [6]: the 28 kDa ligand response domain ($TyrR_{lrd}$) and the 8 kDa operator binding domain. Compared to TyrR of *E. coli*, TyrR of *H. influenzae* lacks the additional N-terminal domain which was found to be critical for positive transcriptional regulation in *E. coli* [7]. Like the central domain of TyrR of *E. coli*, $TyrR_{lrd}$ of *H. influenzae* contains binding sites for both tyrosine and ATP and is assumed to contain the dimerisation determinants.

Recently, Bailey et al. [8] reported on the thermodynamics of operator binding to TyrR of *E. coli*. Using fluorescence quenching of a fluorescein-labelled oligonucleotide, these authors found that ATP strengthened the binding of TyrR to operator DNA. They suggested that the presence of ATP led to a favourable conformational change upon operator binding which generated a more stable protein/DNA complex.

In the current study, we have undertaken fluorescence titration studies to investigate the binding of tyrosine to the $TyrR_{lrd}$ of *H. influenzae* and the dependence of this interaction upon ATP. In addition, secondary structural changes, detected by circular dichroism (CD) spectroscopy, appeared to relate ligand binding to structural effects. Taken together with the results of Bailey et al. [8], the data presented here give a clearer picture of the differences and mutual dependences of affinities for ligands of $TyrR_{lrd}$.

2. Materials and methods

2.1. Expression and purification of $TyrR_{lrd}$

Construction of the expression plasmid, overexpression and purification of $TyrR_{lrd}$ have been described in detail elsewhere [2,6]. For spectroscopical measurements, freshly thawed protein samples were thoroughly dialyzed against NaP_i (50 mM, pH 7.0) containing 200 mM NaCl, 100 μM DTT and 1% glycerol.

Commercially available samples of tyrosine, tryptophan, phenylalanine and ATP (Sigma, St. Louis, MO, USA) were used in the binding studies.

2.2. Fluorescence measurements and data analysis

Steady state fluorescence measurements were recorded on a Perkin Elmer (Beaconsfield, UK) LS50B fluorometer as described previously [9]. In the fluorescence intensity titrations, the emission of a 1 μM $TyrR_{lrd}$ solution upon excitation at 285 nm was recorded over the range of 300–400 nm following the addition of an aliquot of the respective ligand and an equilibration period of 2 min. The use of very concentrated ligand stock solutions ensured a dilution of the protein sample of less than 10%. The slit widths were set at 2 nm and the spectra were recorded with 100 nm/min. A 290 nm cut-off filter was inserted into the emission path to avoid stray light. The samples were stirred during the measurements and the temperature was maintained at 22°C by coupling to an external water bath. After background subtraction, the fluorescence intensity, *F*, was integrated and the mean values resulting from three independent experiments were plotted against the volume-corrected concentration of the added ligand. The resulting binding isotherms were analysed by non-linear regression using the programme Origin (Microcal, Northampton, USA) to the following equation describing a bimolecular association reaction:

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Abbreviations: TyrR, tyrosine repressor; $TyrR_{lrd}$, ligand response domain of TyrR

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$$F = F_i + F_{max}$$

$$\left[\frac{K_d + [TyrR_{lrd}] + [L] - \sqrt{(K_d + [TyrR_{lrd}] + [L])^2 - 4[TyrR_{lrd}][L]}}{2[TyrR_{lrd}]}\right]$$

where F_i is the initial and F_{max} is the maximum fluorescence value, K_d is the dissociation constant and [$TyrR_{lrd}$] and [L] are the total concentrations of $TyrR_{lrd}$ and ligand, respectively. The fitted parameters were F_{max} and K_d. This equation is based on the general solution for a bimolecular association reaction [10].

2.3. CD measurements and analysis

The CD spectra of aqueous $TyrR_{lrd}$ and $TyrR_{lrd}$/ligand complex solutions were recorded in cuvettes with a path length of 0.1 cm on a Jasco J-710 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan). Spectra were collected with a response time of 0.25 s and with a data point resolution of 0.1 nm. Commonly, five scans were averaged to yield smooth spectra. The concentration of $TyrR_{lrd}$ was held constant at 5 μM for all CD measurements. Analysis of the CD spectra with respect to secondary structural elements was accomplished using the programme SELCON [11].

3. Results and discussion

Very limited structural information is available on the NtrC transcription factor family. Intensive sequence comparisons and secondary structure predictions were therefore undertaken as a first step to understand the structure–function relationship of the $TyrR_{lrd}$ of *H. influenzae*. Sequence alignment using BLAST against ProDom domains [12] yielded the highest homology, 39% identity and 56% homology, with the NifA family of transcription factors involved in nitrogen fixation [13]. These proteins are transcription activators and act in concert with the σ^{54} RNA polymerase. Another common feature of the NifA proteins is that they bind ATP. The consensus sequence for ATP binding, (GExGTGKE), is located in the N-terminus of the NifAs [13].

Given the high sequence homology, it was possible to preliminarily identify the ATP binding site in the $TyrR_{lrd}$ of *H. influenzae*. Similarly to the NifAs, ATP binding is presumed to occur near the N-terminus of the protein at the sequence GETGSGKD starting at position 43. According to secondary structure prediction using PredictProtein [14], this sequence is most likely located in an α helix. The overall percentage of secondary structure for $TyrR_{lrd}$ of *H. influenzae* obtained by this numerical method was 45.9% α helix, 13.6% β sheet and 40.5% loop.

In order to experimentally determine the secondary structure content of $TyrR_{lrd}$ of *H. influenzae*, the CD spectra of the protein were recorded. In Fig. 1, the CD spectra of unliganded $TyrR_{lrd}$ as well as spectra obtained of $TyrR_{lrd}$ in complex with different ligands are shown. The high content of α helix becomes immediately obvious from the strong signal at 222 nm (Fig. 1). Upon secondary structure analysis using the SELCON algorithm [11], the overall percentage of secondary structure elements was found to be 42.5% α helix, 17.6% β sheet and 39.9% loop. This result is in very good agreement with the secondary structure prediction obtained by PredictProtein (see above).

Next, the changes in secondary structure induced by various ligands of the $TyrR_{lrd}$ were investigated. Adding a 4-fold molar excess of tyrosine to the $TyrR_{lrd}$ did not affect the CD spectrum of the protein (see insert, Fig. 1). Similarly, the addition of both tyrosine and ATP (10-fold molar excess) had no detectable effect on the CD spectrum of $TyrR_{lrd}$. Interestingly, however, adding ATP in a 10-fold molar excess in the absence of tyrosine resulted in a significant change in the secondary structure of the protein (see Fig. 1). ATP-induced structural changes are a common theme among ATP binding proteins. This fact has recently been documented for the family of chaperones [15]. In the case of $TyrR_{lrd}$, this was also expected, especially because of the location of the ATP binding site within a dominant secondary structural element. The magnitude of this conformational change, however, was found to be dependent upon the presence of tyrosine (see insert, Fig. 1) showing clearly that the binding sites for tyrosine and ATP communicate.

Since the TyrR protein of *E. coli* can also be activated by tryptophan and phenylalanine binding, the effect of these ligands on the CD spectrum of the $TyrR_{lrd}$ was determined in

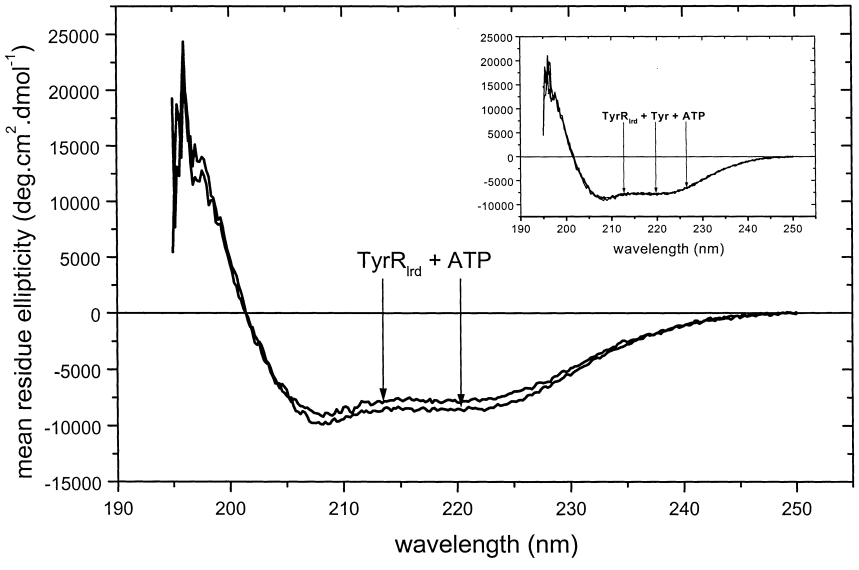

Fig. 1. CD spectra of 5 μM $TyrR_{lrd}$ in the presence of 50 μM ATP and in the presence of 50 μM ATP/20 μM tyrosine (insert). The spectra were corrected for the background signal of buffer plus ligand(s).

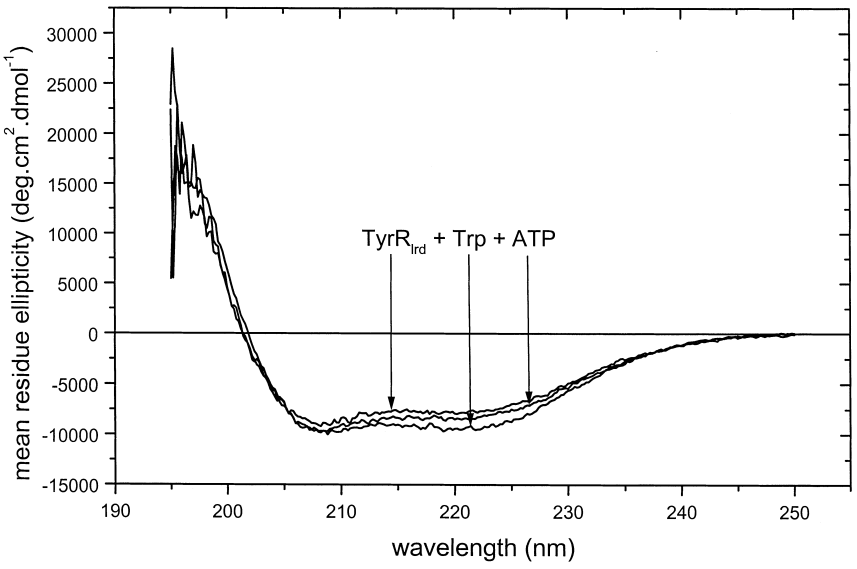

Fig. 2. CD spectra of 5 μM TyrR$_{lrd}$ in the presence of 50 μM ATP/20 μM tryptophan. The spectra were corrected for the background signal of buffer plus ligand(s).

order to investigate possible differences in the induced conformational changes relative to tyrosine (see Fig. 2). In contrast to the study by Argaet et al. [16] in which the authors reported no detectable binding of phenylalanine or tryptophan to the full-length TyrR protein of *E. coli*, we detected a significant change in the TyrR$_{lrd}$ CD spectrum upon the addition of a 4-fold molar excess of either tryptophan or phenylalanine. Distinct secondary structural changes for the two aromatic amino acids were observed independent of whether ATP was present (in a 10-fold molar excess) or absent. Compared with the binary complexes, the ternary complexes consisting of TyrR$_{lrd}$, ATP and aromatic amino acid yielded CD spectra with smaller changes in the secondary structure relative to the unliganded protein. The smallest secondary structural change relative to the unliganded protein was, however, found for tyrosine and tyrosine/ATP binding to TyrR$_{lrd}$ (see insert, Fig. 1). The differences in secondary structure of the ternary complexes, TyrR$_{lrd}$/Tyr/ATP, TyrR$_{lrd}$/Phe/ATP and TyrR$_{lrd}$/Trp/ATP, appear to reflect different modes of repressor activation. As pointed out earlier by Pittard and Davidson [17], the composition of the TyrR/effector complex is decisive for the recognition of a specific transcription unit and for determining whether it is activated or repressed: repression is almost exclusively mediated by tyrosine-activated TyrR, whereas activation is accomplished only by phenylalanine-activated TyrR. Conformational changes due to effector binding are thought to be responsible for tyrosine-mediated repression as well as for phenylalanine-mediated activation, the former leading to cooperative interaction between two TyrR dimers and the latter facilitating cooperativity between one TyrR dimer and RNA polymerase. These differences in effector-induced conformational changes are reflected in the differences among the CD spectra of the respective binary and ternary TyrR$_{lrd}$ complexes (see Figs. 1 and 2).

To further investigate the mutual dependence of the various ligands with respect to binding to TyrR, we determined their binding affinities to TyrR$_{lrd}$ using the fluorescence of the single tryptophan residue at position 223. The maximum emission of TyrR$_{lrd}$ upon excitation at 283 nm occurred at 333 nm, which is typical for a rather solvent-shielded tryptophan chromophore. Adding the ligands ATP, tyrosine or phenylalanine resulted in a quenching of the fluorescence intensity and led to a 2 nm red-shift of the maximum emission. This can be interpreted as a conformational change of the Trp-223 microenvironment upon ligand binding [18]. Binding of tryptophan to TyrR$_{lrd}$ could not be investigated by this method because of the strong fluorescence of this ligand which rendered background correction impossible. From the saturation curves obtained by titrating the TyrR$_{lrd}$ with increasing amounts of ligand, the K_d value for the respective interaction was calculated (see Table 1).

Assuming a stoichiometry of 1 mol ligand per mol TyrR$_{lrd}$ monomer [16], the highest K_d value and thus the lowest affinity was found for the interaction of ATP with TyrR$_{lrd}$ in the absence of tyrosine (see Fig. 3, curve A). The affinity for ATP could be raised by first pre-incubating TyrR$_{lrd}$ with a 4-fold molar excess of tyrosine, then titrating with increasing amounts of ATP (see Fig. 3, curve B). This result stands in contrast to the higher affinity of tyrosine for the TyrR$_{lrd}$ in the absence of ATP compared to its affinity in the presence of a 10-fold molar excess of ATP (see Table 1). The K_d for the binding of phenylalanine to TyrR$_{lrd}$ was found to be 10 times higher than that of tyrosine (see Table 1). In the absence of ATP, the K_d of phenylalanine also increased although the difference in the respective dissociation constants was not as large as for tyrosine (see Table 1). Our observations are in agreement with the lack of a role for ATP in phenylalanine-

Table 1
Dissociation constants for TyrR$_{lrd}$/ligand interactions. Data were analysed as described in Section 2

Receptor	Ligand	K_d (10^{-6} M)
TyrR$_{lrd}$	Tyr	0.043 ± 0.024
TyrR$_{lrd}$/ATP	Tyr	1.089 ± 0.146
TyrR$_{lrd}$	Phe	0.573 ± 0.072
TyrR$_{lrd}$/ATP	Phe	1.062 ± 0.135
TyrR$_{lrd}$	ATP	2.396 ± 0.242
TyrR$_{lrd}$/Tyr	ATP	1.198 ± 0.054

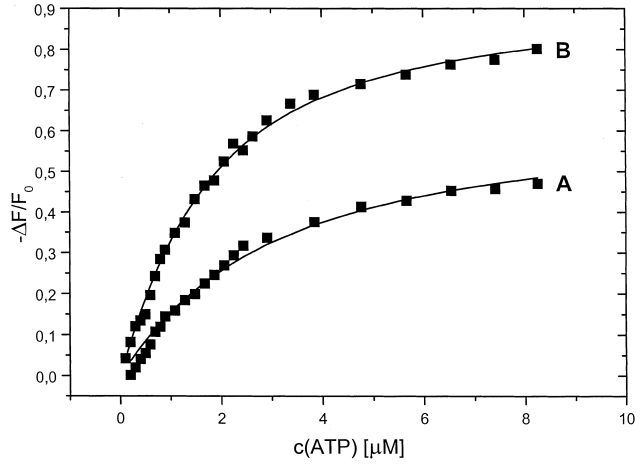

Fig. 3. Binding isotherms for the interaction of $TyrR_{lrd}$ with ATP in the absence (curve A) and in the presence (curve B) of tyrosine. The changes in fluorescence are plotted as the normalised change in fluorescence ($-\Delta F/F_0$, where F_0 is the initial fluorescence intensity before addition of ligand) vs. the concentration of ATP (μM). The data represent mean values of three independent experiments and were fitted as described in Section 2.

mediated activation of TyrR-controlled transcription units [17].

In conclusion, we propose a model for the ligand-induced activation of TyrR based on our CD secondary structure and fluorescence binding studies. In a multi-component transcription complex, the critical activating molecule is assumed to be the one with the highest affinity for its receptor which is commonly also rate-limiting due to its low concentration such as in the situation of starvation. Tyrosine has by far the lowest K_d for the interaction with its receptor (Table 1). Since the affinity for tyrosine decreases in the presence of ATP, whereas the affinity for ATP is increased in the presence of tyrosine, the mutual dependence of ligand binding to the $TyrR_{lrd}$ of *H. influenzae* in vitro becomes obvious. This is also reflected in the secondary structural changes upon ligand binding. ATP itself induced a change in the overall secondary structure which was reversed by the addition of tyrosine, whereas no changes occurred when the order of ligand addition started with tyrosine followed by ATP (see Fig. 1). The differences in dissociation constants as well as in conformational changes found for the other aromatic amino acids, phenylalanine and tryptophan, reflect thermodynamic and structural fine tuning that are the hallmarks of transcriptional regulators such as TyrR. These results, viewed in the light of the findings of Bailey et al. [8] showing that DNA binds more strongly to TyrR in the presence of ATP, prove that the nucleotide is a conformational co-activator of TyrR. How this mutual dependence of ligand binding to TyrR comes into play in vivo, where the concentration of ATP is reported to be 3 mM [19] and TyrR is thus always saturated with the nucleotide, remains to be clarified.

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References

[1] Pittard, A.J. (1996) in: *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology (Neidhardt, F.C., Curtiss, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M. and Umbarger, H.E., Eds.), pp. 458–484, ASM Press, Washington, DC.
[2] Zhu, Q., Zhao, S. and Somerville, R.L. (1997) Protein Expr. Purif. 10, 237–246.
[3] Cui, J. and Somerville, R.L. (1993) J. Biol. Chem. 268, 5040–5047.
[4] Yang, J., Ganesan, S., Sarsero, J. and Pittard, A.J. (1993) J. Bacteriol. 175, 1767–1776.
[5] Cui, J., Ni, L. and Somerville, R.L. (1993) J. Biol. Chem. 268, 13023–13025.
[6] Zhao, S. and Somerville, R.L. (1999) J. Biol. Chem. 274, 1842–1847.
[7] Cui, J. and Somerville, R.L. (1993) J. Bacteriol. 175, 303–306.
[8] Bailey, M.F., Davidson, B.E., Haralambidis, J., Kwok, T. and Sawyer, W.H. (1998) Biochemistry 37, 7431–7443.
[9] Brunner, K., Tortschanoff, A., Hemmens, B., Andrew, P.J., Mayer, B. and Kungl, A.J. (1998) Biochemistry 37, 17545–17553.
[10] Nomanbhoy, T.K. and Cerione, R. (1996) J. Biol. Chem. 271, 10004–10009.
[11] Sreerama, N. and Woody, R.W. (1993) Anal. Biochem. 209, 32–44.
[12] Corpet, F., Gouzy, J. and Kahn, D. (1998) Nucleic Acids Res. 26, 323–326.
[13] Fischer, H.-M. (1994) Microbiol. Rev. 58, 353–386.
[14] Rost, B. and Sander, C. (1993) J. Mol. Biol. 232, 584–599.
[15] McKay, D.B. and Ha, J.H. (1995) Biochemistry 34, 11635–11644.
[16] Argaet, V.P., Wilson, T.J. and Davidson, B.E. (1994) J. Biol. Chem. 269, 5171–5178.
[17] Pittard, A.J. and Davidson, B.E. (1991) Mol. Microbiol. 5, 1585–1592.
[18] Ringhofer, S., Kallen, J., Dutzler, R., Billich, A., Visser, A.J.W.G., Scholz, D., Steinhauser, O., Schreiber, H., Auer, M. and Kungl, A.J. (1999) J. Mol. Biol. 286, 1147–1159.
[19] Bochner, B.R. and Ames, B.N. (1982) J. Biol. Chem. 257, 9759–9769.