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The Influence of ATP on the Association and Unfolding of the Tyrosine Repressor Ligand Response Domain of *Haemophilus influenzae*

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The secondary structure of the ligand response domain of the *Haemophilus influenzae* tyrosine repressor, $TyrR_{lrd}$, was investigated using CD spectroscopy which revealed 42.5% α-helix, 17.6% β-sheet, and 39.9% loops. Quaternary structure analysis by fluorescence anisotropy showed that $TyrR_{lrd}$ is monomeric at a concentration of 100 nM to 2 μM but that the protein readily dimerizes in the presence of its natural ligand ATP. Equilibrium unfolding studies of $TyrR_{lrd}$ using guanidinium hydrochloride suggested a two-state model with no detectable stable intermediates. The unfolding transition monitored by CD spectroscopy was responsive to tyrosine and ATP resulting in a shift to higher denaturant concentrations in the presence of these ligands. Differential scanning calorimetry yielded melting temperatures, T_m, of 51.15 and 58.07°C for the unliganded and for the ATP-liganded protein, respectively. ATP is thus proposed to be a major structural cofactor for the molecular architecture of $TyrR_{lrd}$.

The tyrosine repressor (TyrR) proteins belong to the NtrC superfamily of prokaryotic transcription factors. Their role is to control the expression of several genes involved in the biosynthesis and transport of aromatic amino acids (1, 2). Their exact mode of action is still unclear since these proteins are able to selectively up- or down-regulate the transcription of different target genes. TyrR of *E. coli* (513 amino acids) consists of three domains, the N-terminal, the central and the DNA binding domain. The central domain (residues 191–467) contains both the tyrosine binding site and at least one binding site for ATP (3, 4). The exact role of ATP remains uncertain although TyrR was shown to exhibit weak ATPas activity (5).

Based upon limited proteolytic cleavage, TyrR of *H. influenzae* (318 amino acids) was found to consist of only two domains (6): a 28 kDa ligand response domain ($TyrR_{lrd}$) and an 8 kDa DNA binding domain. Compared to TyrR of *E. coli* the *H. influenzae* protein lacks the N-terminal domain known to be critical for positive transcriptional regulation (7). $TyrR_{lrd}$ of *H. influenzae*, like the central domain of TyrR of *E. coli*, contains binding sites for tyrosine and ATP as well as one or more segments that are important for the self-association of monomers into homodimers.

The folding of multimeric proteins requires the orderly and specific acquisition of secondary, tertiary and quarternary structure. Despite the wealth of information on the formation of secondary and tertiary structure in monomeric proteins, little is known about how these reactions are coupled with association reactions that occur during quaternary structure formation (8). Recently, equilibrium unfolding studies of dimeric tryptophan repressor and tetrameric lactose repressor revealed a two-state model for both proteins (9, 10). This implies that during unfolding the disruption of the monomer-monomer interface and monomer unfolding are concerted events.

In the case of TyrR the situation is even more complicated, owing to the fact that ATP might influence the quaternary structure of the protein and could thus have an impact on its unfolding behavior. We have recently shown that ligand binding to the 257 amino acid ligand response domain of *H. influenzae* TyrR, $TyrR_{lrd}$, influenced the secondary structure of the protein. (11). Here we present evidence that $TyrR_{lrd}$ readily dimerizes in the presence of ATP but that both monomeric and dimeric $TyrR_{lrd}$ exhibit equilibrium unfolding transitions with no stable intermediates.

Abbreviations used: TyrR, tyrosine repressor; $TyrR_{lrd}$, ligand response domain of TyrR; Gua · HCl, guanidinium hydrochloride; CD, circular dichroism; DSC, differential scanning calorimetry.

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MATERIALS AND METHODS

Expression and purification of $TyrR_{lrd}$. Construction of the expression plasmid, overexpression and purification of $TyrR_{lrd}$ have been described in detail elsewhere (2, 5). For spectroscopic measurements, freshly thawed protein samples were thoroughly dialyzed against NaP_i (50 mM, pH 7.0) containing 200 mM NaCl, 100 μM DTT and 0.1% Trasylol (Bayer, Leverkusen, Germany). Commercially available samples of tyrosine and ATP (Sigma, St. Louis, MO) were used in all experiments.

Fluorescence anisotropy measurements. Steady state fluorescence measurements were recorded on a Perkin Elmer (Beaconsfield, UK) LS50B fluorometer as described previously (11, 12). The fluorescence anisotropy of $TyrR_{lrd}$ was collected at an emission wavelength of 333 nm with the excitation wavelength set at 283 nm. Changes in quaternary structure were followed by varying the protein concentration in the presence or absence of ATP. The fluorescence anisotropy is defined as

$$r = (I_{VV} - G \cdot I_{VH})/(I_{VV} + 2G \cdot I_{VH}),$$

where I_{VV} is the fluorescence intensity recorded with vertically aligned excitation and emission polarizers, and I_{VH} is the fluorescence intensity recorded with the emission polarizer horizontally aligned. The G factor is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light, $G = I_{HV}/I_{HH}$. For each point in the anisotropy experiments, the signal was recorded over 60 s and the mean r values were plotted against the $TyrR_{lrd}$ concentration.

CD measurements and analysis. Far-UV CD spectra were recorded on a Jasco J-710 spectropolarometer (Japan Spectroscopy, Tokyo, Japan) as described previously (11). For the CD unfolding experiments, the signal at 222 nm of a 5 μM $TyrR_{lrd}$ solution was collected for each guanidinium hydrochloride (Gua · HCl) concentration over 60 s in cuvettes with a path length of 0.1 cm. The observed time course was averaged and corrected for the background signal of the buffer and of the ligands ATP and tyrosine. The resulting data were plotted against the concentration of Gua · HCl. The unfolding transition was identified as midpoint between the initial and final value by sigmoidally fitting the data to the Boltzmann equation using the program Origin (Microcal, Northampton, MA).

Differential scanning calorimetry. Thermal unfolding measurements were performed using a MicroCal VP DSC calorimeter (Microcal, Northampton, MA). The concentration of $TyrR_{lrd}$ was adjusted to 8.5 μM and the samples were extensively degassed prior to the experiments. The temperature scans were recorded from 10 to 70°C with a heating rate of 1°C/min. Data analysis was performed using the Origin (Microcal, Northampton, MA) software after subtraction of the buffer baseline and after defining a progress-baseline for the native and the heat-denaturated state. The processed data were fit to a model for a non-two-state transition to determine the transition temperature T_m and ΔH_{cal}.

RESULTS AND DISCUSSION

Recording of the fluorescence anisotropy, r, is commonly used to study the quaternary structure and association state of (biological) macromolecules (13). For this purpose, either an intrinsic chromophore must be present in the molecule or an extrinsic chromophore must be employed. The $TyrR_{lrd}$ of *H. influenzae* contains a strong emitting fluorophore, namely the tryptophan residue at position 223, which was recently shown to be sensitive to ligand binding (11).

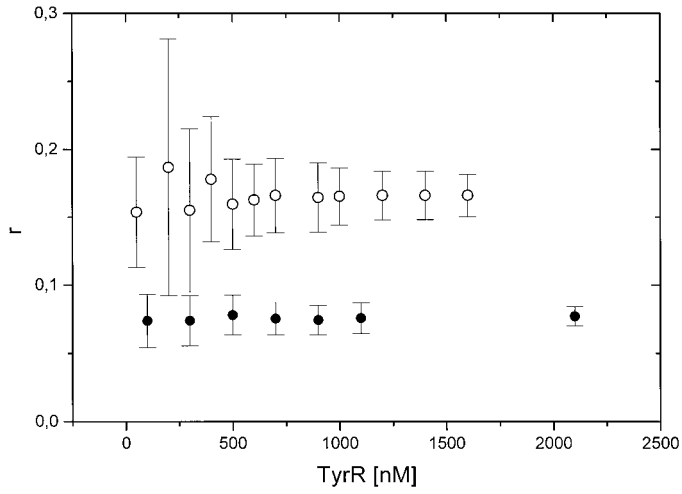

FIG. 1. Fluorescence anisotropy of increasing concentrations unliganded $TyrR_{lrd}$ (●) and in the presence of 10 μM ATP (○). Each data point represents the mean value obtained from averaging the value of r determined over 60 s.

As shown in Fig. 1, the fluorescence anisotropy of $TyrR_{lrd}$ was constant over a protein concentration range of 200 nm to 2 μM. The average value of the fluorescence anisotropy, $r = 0.0754$, is indicative for monomeric $TyrR_{lrd}$. In the presence of ATP, however, the value of r increased to 0.16574 suggesting association, most probably homodimerization, of $TyrR_{lrd}$. It is likely that ATP influences the dimerization interface of $TyrR_{lrd}$ by inducing a conformational change, an indication for which is the recently published difference in the CD spectra of the $TyrR_{lrd}$/ATP complex relative to the unliganded protein (10). Little is known about the general role of ATP as a conformational activator of protein association reactions. In the case of hexokinase, ATP reverses dimerization mediated by glucose 6-phosphate (14) which points to a structural role for ATP at the dimer interface of this protein. The effect of ATP on the association state of $TyrR_{lrd}$ might thus suggest a new function for this nucleotide. For the subsequent unfolding studies of $TyrR_{lrd}$ this meant that a "dimerization switch" was at hand, thus avoiding the generation of monomeric mutant proteins (15), with which the denaturation of monomeric and dimeric $TyrR_{lrd}$ could be individually investigated. The influence of the activating ligand tyrosine on the dimerization behavior of $TyrR_{lrd}$ could not be investigated by this method because of the high intrinsic fluorescence signal of this ligand.

Monomeric $TyrR_{lrd}$ was found to be well structured as revealed by far-UV CD spectroscopy (see Fig. 2). A highly α-helical structure for the protein was detected by SELCON analysis (16) of the CD data yielding 42.5% α-helix, 17.6% β-sheet and 39.9% loop. This was in excellent agreement with secondary structure predictions for $TyrR_{lrd}$ by Predict Protein (17) which gave 45.9% α-helix, 13.6% β-sheet and 40.5% loop (see inset

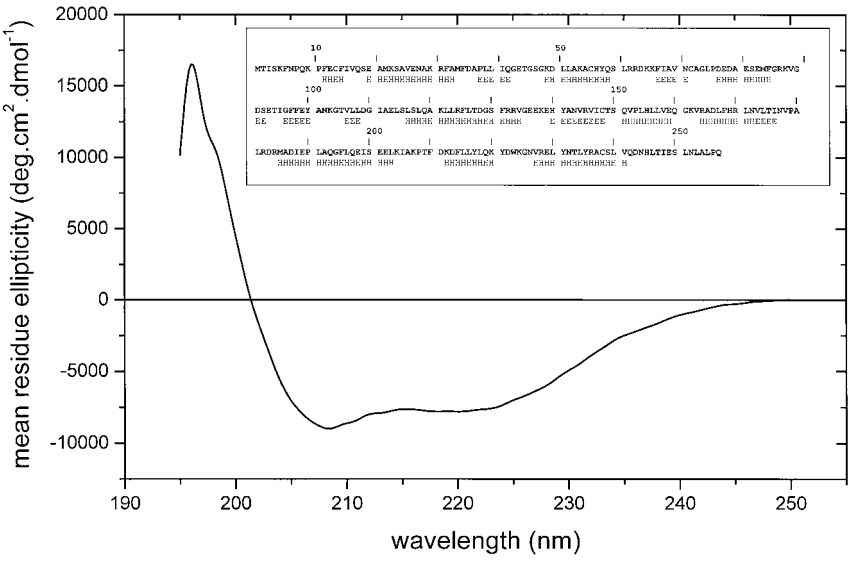

FIG. 2. CD spectrum of $TyrR_{lrd}$ in 50 mM NaPi buffer (pH 7.0). Three scans were run to obtain smooth data. (Inset) Secondary structure prediction obtained by Predict Protein (17).

in Fig. 2). A high α-helical content is a common structural feature of prokaryotic transcription factors such as the tryptophan repressor, TrpR, which is purely α-helical, and lactose repressor, LacR, which consists of 33.3% α-helix and 14.4% β-sheet. Whether this common characteristic would lead to a similar unfolding behavior was investigated next.

Unfolding of $TyrR_{lrd}$ was accomplished by adding the chaotropic reagent guanidinium hydrochloride (Gua · HCl). Because of the high α-helical content of $TyrR_{lrd}$, denaturation was followed by recording the CD signal at 222 nm (18) which is displayed in Fig. 3. The unfolding transition of a 5 μM solution of $TyrR_{lrd}$ occurred at 1.5 M Gua · HCl. Since no stable intermediates were detected, unfolding of $TyrR_{lrd}$ is interpreted as a two-state model. This is quite similar to the unfolding behavior of the tryptophan and the lactose repressor (9, 10). The concentrations of Gua · HCl required for the unfolding transition of $TyrR_{lrd}$ were found to be higher in the presence of a 4-fold molar excess tyrosine (1.6 M) and ATP (1.8 M) (see Fig. 3). The impact of ligand binding on the native structure of $TyrR_{lrd}$ is reflected in the lower initial CD signal at 222 nm in the presence of these ligands compared to the unliganded protein. Despite this influence on the secondary structure of $TyrR_{lrd}$, the ligands obviously induced a more compact structure which was relatively resistant to denaturation. needs higher concentrations of denaturant to become unfolded. The unfolding of $TyrR_{lrd}$ was irreversible both in the presence and in the absence of ligands.

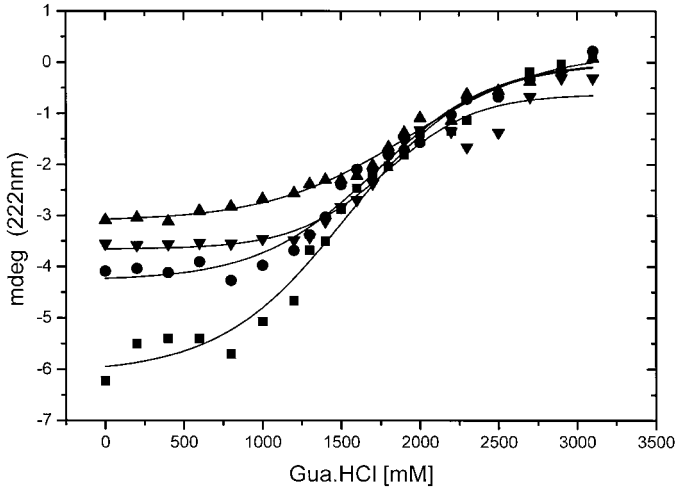

FIG. 3. Unfolding of $TyrR_{lrd}$. The CD signal at 222 nm of $TyrR_{lrd}$ (■), of $TyrR_{lrd}$ in complex with tyrosine (●), of $TyrR_{lrd}$ in complex with ATP (▼), and of $TyrR_{lrd}$ in complex with tyrosine and ATP (▲) is plotted against increasing concentrations of Gua · HCl.

In order to study the thermal denaturation of $TyrR_{lrd}$, differential scanning calorimetry was employed (see Fig. 4). By this method, a melting temperature T_m = 51.15°C was found for unliganded $TyrR_{lrd}$ corresponding to a calorimetric enthalpy of unfolding ΔH_{cal} = 17.6 kcal/mol. In the presence of tyrosine, the thermal transition of $TyrR_{lrd}$ became significantly broader and ΔH_{cal} increased to 30.8 kcal/mol (see Fig. 4). This refers to protein–ligand interactions which add to the unfolding enthalpic signal. The T_m of $TyrR_{lrd}$ remained almost unchanged at 50.68°C in the presence of tyrosine. Upon addition of ATP, the melting temperature of $TyrR_{lrd}$ shifted significantly to 58.07°C which was associated with a ΔH_{cal} = 23.5 kcal/mol and a very broad unfolding transition (see Fig. 4). ATP obviously not only mediated additional intermolecular interac-

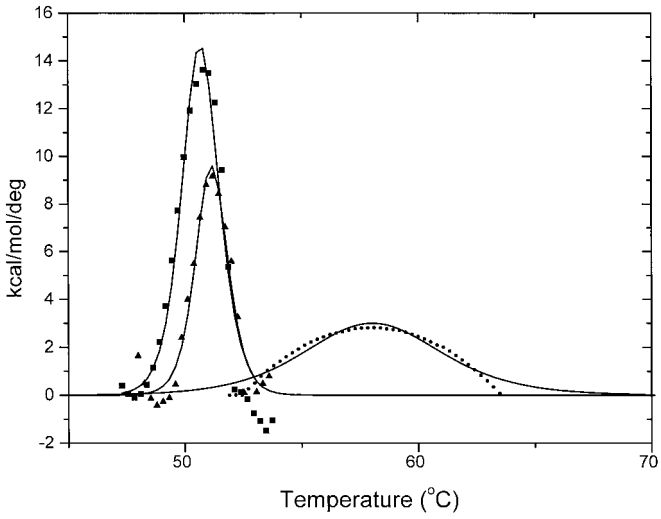

FIG. 4. Thermal unfolding of $TyrR_{lrd}$ (▲), of $TyrR_{lrd}$ complexed with tyrosine (■), and of $TyrR_{lrd}$ complexed with ATP (●) monitored by DSC. Solid lines represent best fits of the data according to a non-two-state transition.

tions, detected as an increase of ΔH_{cal} relative to the unliganded protein, but also modified the aggregation state of $TyrR_{lrd}$ causing a shift in T_m. Especially the very broad transition found for the ATP complex is proposed to be indicative of a distribution of unfolded states which may be further corroborated by the presence of partly unfolded monomeric and dimeric forms of $TyrR_{lrd}$.

In conclusion, we have shown that the association state of $TyrR_{lrd}$ is influenced by the presence of the natural ligand ATP. In addition, unfolding of the protein, which was shown to follow a two-state model, was found to respond to ATP, indicating that this nucleotide has a conformationally stabilizing effect in addition to its ability to induce homodimerization.

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